Daniela Pereira Soares

Licenciada em Engenharia Química e Bioquímica

Valorization of tomato waste with subcritical water extraction

Dissertação para obtenção do Grau de Mestre em
Engenharia Química e Bioquímica

Orientador: Professor Pedro Miguel Calado Simões, DQ – FCT/UNL

Coorientadora: Professora Susana Filipe Barreiros, DQ – FCT/UNL

Júri:

Presidente: Professor Doutor Mário Fernando José Eusébio

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“It is our choices that show what we truly are, far more than our abilities”

J. K. Rowling

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Acknowledgements

Primeiramente gostaria de agradecer ao Professor Pedro Simões, à Professora Susana Barreiros e ao Dr. Alexandre Paiva, por me terem recebido de braços abertos no laboratório e por estarem sempre disponíveis para qualquer dúvida. À Professora Margarida Gonçalves por me ter acolhido no seu laboratório na fase final da minha tese, e por se ter disponibilizado a me ajudar.

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Abstract

World energy demand is increasing rapidly, and the search for new renewable and clean technologies has been a priority for the agro-industrial sector. Greenhouse gases emission and pollution has had great concern all over the globe. Biomass is the only renewable resource abundant enough to be used as an energy source. Tomato waste is a common agro-industrial residue, and it is abundantly produced by tomato processing industries. Biorefineries can use agro-industrial residues to produce added-value residues, increasing its value.

The aim of this work is to evaluate the possibility of tomato waste valorization from the tomato processing industries, through the extraction/hydrolysis of carbohydrates and phenolic compounds with antioxidant activity, using a clean and efficient technology, subcritical water extraction. ScW extraction was performed at 100 bar, a constant water flow of 12 mL/min and a range of temperatures until 250 ºC, by extracting different compounds at the target temperatures of 140 ºC, 190 ºC and 250 ºC.

Tomato waste (TW) was chemically characterized, having a high content in carbohydrates (around 38 wt.%), of which ca. 52% was non-structural carbohydrates. Main carbohydrate monomers identified by HPLC, were glucose and fructose. Tomato waste has also a high content in lignin (19.2 wt.%) and protein (15.2 wt.%). Phenolic content was about 0.6 wt.%. Ash, lipid, and water content were also determined.

Tomato waste was initially submitted to supercritical CO_2 extraction to remove most of its lipid and lycopene content. The residue that was left from the previous extraction, labelled as defatted TW (DTW), was subjected to subcritical water (ScW) extraction, with a high yield of 67% and a mass conversion of 86%. ScW residue and ScW extracts were chemically characterized. 73% of the total amount of carbohydrates were recovered in the ScW extracts, and 11% in the ScW residue, making a total of 84% of carbohydrate recovery in the process. A higher amount of phenolic compounds was recovered in the ScW extracts than quantified in the original material, indicating that the subcritical water treatment allowed access to compounds not recovered in the hydro-alcoholic extraction. The antioxidant activity of each extract was evaluated, where the extract with a higher antioxidant activity, obtained at 250 ºC, had an inhibition percentage of 54%.

The main goals of this work were achieved successfully. Carbohydrate and phenolic compounds were extracted with subcritical water with high biomass recovery, and antioxidant activity was confirmed in ScW extracts, demonstrating the possibility of tomato waste valorization.

Keywords: Agro-industrial residues, antioxidant activity, biomass, carbohydrates, phenolic compounds, subcritical water, tomato waste.

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Resumo

A necessidade de energia a nível global está a aumentar rapidamente, e a procura por tecnologias limpas e renováveis tem sido uma prioridade para o setor agroindustrial. A emissão de gases de efeito de estufa e a poluição têm gerado uma grande consciencialização em todo o mundo. A biomassa é o único recurso renovável em abundância suficiente para ser utilizado como fonte de energia. O repiso do tomate é um resíduo agroindustrial comum, que é abundantemente produzido durante o processamento do tomate pelas indústrias desse setor. As bio-refinarias conseguem utilizar resíduos agroindustriais para produzir produtos com valor acrescentado.

O objetivo deste trabalho é avaliar a possibilidade de valorização do repiso do tomate proveniente das indústrias de processamento, através da extração/hidrólise de carboidratos e compostos fenólicos com atividade antioxidante, utilizando uma tecnologia limpa e eficiente, a extração com água subcrítica. A extração com água subcrítica foi realizada a 100 bar, um caudal de água constante de 12 mL/min e numa gama de temperaturas até 250 ºC, extraindo diferentes compostos a temperaturas alvo, 140 ºC, 190 ºC e 250 ºC.

O repiso do tomate (TW) foi caracterizado quimicamente, tendo um alto conteúdo em carboidratos (cerca de 38% m/m), dos quais cerca de 52% são carboidratos não estruturais. Os monómeros de carboidratos identificados por HPLC são a glucose e frutose. O repiso do tomate apresenta também uma elevada quantidade de lignina (cerca de 19,2% m/m) e proteína (15,2% m/m). O conteúdo em compostos fenólicos é cerca de 0,6% m/m. Os conteúdos de cinzas, lípidos e água foram também determinados.

O repiso do tomate foi inicialmente submetido a uma extração com CO_2 supercrítico para remover os lípidos e licopeno. Ao resíduo resultante desta extração deu-se o nome de repiso do tomate desengordurado (DTW). Este resíduo foi submetido a extração com água subcrítica (ScW), com um alto rendimento de 67% e uma conversão de matéria de 86%. O resíduo e os extratos da extração foram caracterizados quimicamente. 73% da quantidade total de carboidratos foram recuperados nos extratos ScW, e 11% no resíduo ScW, perfazendo um total de 84% de recuperação de carboidratos no processo. Uma quantidade superior de carboidratos foi recuperada nos extratos ScW do que os quantificados no material original, indicando que o tratamento com água subcrítica permitiu o acesso a compostos que não foram recuperados na extração hidroalcoólica. A atividade antioxidante de cada extrato foi avaliada, onde o extrato com maior atividade, a 250 ºC, tinha uma inibição de 54%.

Os objetivos principais deste trabalho foram atingidos com sucesso. Os compostos fenólicos e carboidratos foram extraídos com água subcrítica com uma alta recuperação de biomassa, e a atividade antioxidante foi confirmada nos extratos, demonstrando que é possível a valorização do repiso do tomate.

Palavras-Chave: Água subcrítica, atividade antioxidante, biomassa, carboidratos, compostos fenólicos, repiso do tomate, resíduos agroindustriais.

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Abbreviations and Symbols

CE	Catechin Equivalent
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTW	De-fatted Tomato Waste
GAE	Gallic Acid Equivalent
GC-MS	Gas Chromatography-Mass Spectrometry
GE	Glucose Equivalent
HPLC	High Performance Liquid Chromatography
ScW	Subcritical Water
SPE	Solid Phase Extraction
TW	Tomato Waste

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Chapter 1

Introduction and Motivation

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1.1. Waste Management

1.1.1. Biorefinery Concept

The world's future relies on how energy resources are managed nowadays. Energy, water, clean air, food, and resources are required to maintain the current lifestyle, and a balance between them must be found. Industrial growth in the last decades leads to higher energy needs which currently implies polluting the air, land, and oceans. [1]

Energy resources include all forms of fuels used and can be divided into renewable and non-renewable. Non-renewable energy comes from sources that, by definition, will not be replenished in our lifetime or that will run out. Most non-renewable energy sources are from fossil fuels, such as natural gas, oil, and coal. There is also nuclear energy, where nuclear power plants produce energy through nuclear fission and fusion. Non-renewable energy sources are very efficient, easier to find, and simpler to transport. However, they will run out, and the process releases carbon dioxide, directly linked to global warming and environmental pollution. [2] On the other hand, renewable energy is a natural resource that can replace itself rapidly and that, unlike non-renewable energy sources, will not run out. This category includes wind and solar energy, hydropower, geothermal energy, and biomass. Renewable energy sources reduce water and air pollution and global warming emissions, marking a clearer path towards greener environmental policies. [3]

According to the U.S. Energy Information Administration (EIA), in 2019, about 100.2 quadrillion Btu (105.7 quadrillion kilojoules) of energy were consumed in the U.S. alone. Prospects expect that renewable energy will become the leading source of primary energy consumption by 2050, increasing by 3% per year between 2018 and 2050. [4]

Although renewable energy sources are expected to have the highest growth, other energy forms will also increase due to energy demand. [4] Global energy demand is expected to increase by 20%, reaching 675 quadrillion BTU (712 quadrillion kilojoules) in 2040. This reflects a growing population and increasing prosperity, and about 50% is dedicated to industrial activity. Figure 1.1 shows the variation of energy source consumption worldwide up until 2040. By then, oil is still expected to be the most consumed energy source, even though renewable energies demonstrate a strong growth. [5]

Global warming has been and will continue to be a major concern worldwide. IPCC Special Report on Global Warming, released on October 8th, 2018, estimates that human activities have caused a 1 ^{o}C increase comparing to pre-industrial levels. Countermeasures must be taken to restore or prevent the gradual rising of the world's temperature. Reducing CO_2 emissions has a significant role in energy transition. Due to governments' efforts in that direction, energy-related CO_2 emissions in the IRENA Remap Reference case between 2015 and 2050 have declined from a projected 1 380 Gigatons based on 2017 analysis to 1 230 Gigatons in 2018, a drop of 1%.

However, these measures are not yet reflected with real numbers, having increased 1.4% in 2017. [6]

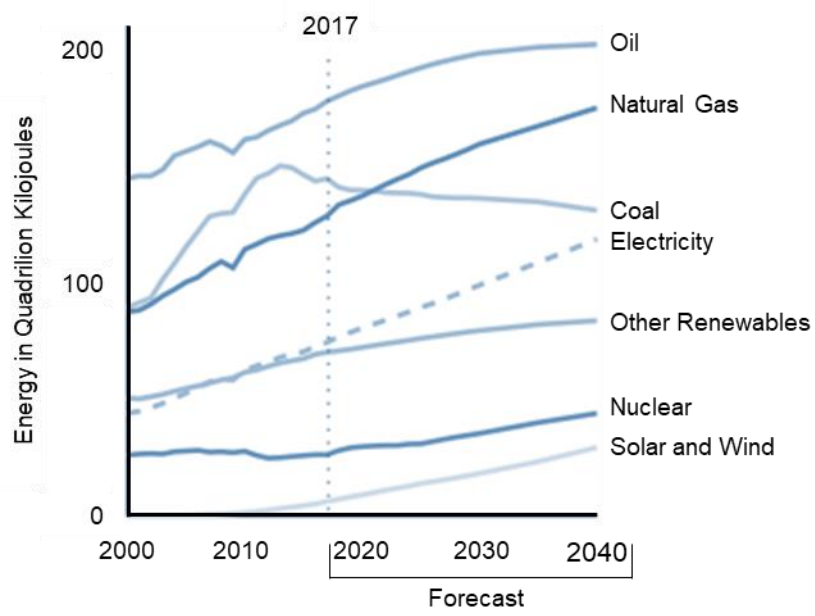

Figure 1.1: Energy consumption by energy source worldwide through the years and forecast (Adapted from [5]).

Renewable energies must get more ground to reduce greenhouse gases emissions. Biomass is the most important renewable energy source in the world. It is all non-fossil organic material that has intrinsic chemical energy content. It is the only other naturally occurring energy source containing carbon that is big enough to replace fossil fuels. Being a renewable source of energy, it has the advantage of never running out, comparing to fossil fuels. This kind of energy has been getting more attention in the last decades, for being more efficient and more economical to produce. Also, biomass combustion produces fewer nitrogen oxides and sulfur dioxide than fossil fuels, being less pollutant than fossil fuels. [7]

Biomass is all the organic matter from wood, waste, such as solid waste or animal, plant and manufacturing wastes, and alcohol fuels, like ethanol, from corn and other grains. Industrialized countries have over 1 500 million hectares of crop forest and woodland, of which 460 million hectares are cropland. Extracting energy from biomass is an antique practice, from the time when people burnt wood for heat and light. Energy is one of the most basic human needs, and it is directly connected to economic growth. The use of biomass to produce energy is a greener and environmentally friendly alternative to the classic fossil fuels utilization. [8]

Classic refineries use petroleum as feedstock, producing various products that are then sold as final products or transformed into added-value products, like plastics and fibers. About 17% of the volume of the products are classified as chemicals. This implies that 17% of chemicals are petroleum dependent. If these same chemicals could be produced from biomass in a biorefinery, a positive environmental impact would be obtained. [9]

A biorefinery uses a combination of processes to produce fuel, energy, and value-added products from biomass. Figure 1.2 is a schematic representation of the biomass concept. [10]

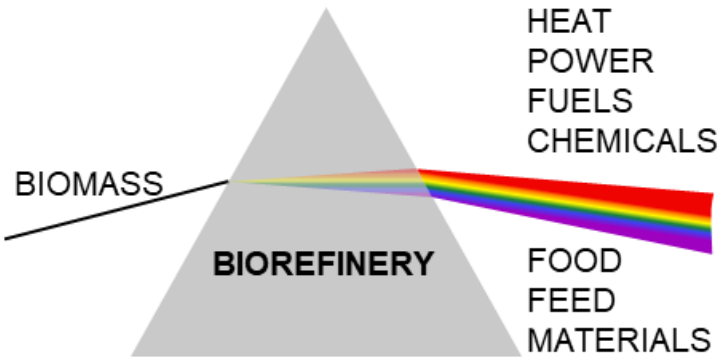

Figure 1.2: Biomass concept representation (Adapted from [10]).

Biorefineries' main driver is sustainability, and its assessment should consider the entire life cycle, going through the environmental, economic, and social sustainability of its value chain. A biorefinery processes biomass into an extensive range of products. Biorefinery feedstocks are quite various, from agriculture, forestry, aquaculture, and residues from industry, including, among others, agriculture crops and organic residues. [10]

Different biorefineries have been studied, each having its characteristics and different outlets. The most common biorefineries are the conventional ones, while “whole crop”, “oleochemical”, “Lignocellulosic feedstock”, “marine” and “green biorefineries” are still in development. Lignocellulosic feedstock biorefinery refines lignocellulosic biomass such as wood and straw into intermediate products, like cellulose, hemicellulose, and lignin. These intermediate products are then used to produce a new range of products and bioenergy. This type of biomass is expected to become the most important source of biomass in the future while having a moderate cost and high availability. [10]

The use of new feedstocks in biorefineries will be adding value to different residues while producing a range of biobased products and bioenergy. This could make significant contributions to sustainable development, reinforcing the economic position of several market sectors. However, efficiency should be maximized since biomass is limited, combining the different biorefineries purposes effectively. [10] Biomass feedstock is a renewable energy source, and its importance will increase under the governments' pressure to reduce greenhouse gases emissions. Comparing to fossil fuels, biorefineries can be more efficient and have lower pollution emissions. [7]

1.1.2. Agro-industrial residues

Agro-industrial residues are the most abundant resource on Earth. They are the most common biomass type for their low-cost price and renewability. Most agro-industrial wastes are underutilized and untreated, being disposed of in landfills or burned, causing a severe disposal

problem. [11] These residues are getting more attention for quality control, their high nutritional potential, and are being categorized as agro-industrial by-products. Agro-industrial wastes can be divided into agriculture and industrial residues. Agriculture residues include field and processing residues, such as leaves, seeds, stems, stalks, molasses, bagasse, roots, husks, etc. Through food processing industries like fruit, juice, meat, chips industries, organic residues are produced that can be used for different energy sources. [11]

Several studies have reported that agro-industrial wastes can be used for different purposes, such as natural antimicrobials (pomegranate and lemon peels, and walnut husks) and bio-based products like biofertilizers and bioenergy. These residues usually contain a large amount of proteins, carbohydrates, and minerals, varying their composition depending on the type of residue, making them raw materials for new products instead of “wastes”. Table 1.1 shows the composition of most common agro-industrial wastes in cellulose, hemicellulose, and lignin, that can produce biogas, bio-ethanol, and others, through biochemical digestion. [11]

Table 1.1: Composition of some agro-industrial wastes (Adapted from [11])

Agro-Industrial Waste	**Chemical Composition (% w/w)**		
	Cellulose	**Hemicellulose**	**Lignin**
Sugarcane Bagasse	30.2	56.7	13.4
Sugar Beet	26.3	18.5	2.5
Sawdust	45.1	28.1	24.2
Corn Stalks	61.2	19.3	6.9
Cotton Stalks	58.5	14.4	21.5
Soya Stalks	34.5	24.8	19.8
Sunflower Stalks	42.1	29.7	13.4
Barley Straw	33.8	21.9	13.8
Oat Straw	39.4	27.1	17.5
Rice Straw	39.2	23.5	36.1
Wheat Straw	32.9	24.0	8.9
Coffee Skin	23.8	16.7	28.6
Orange Peel	9.2	10.5	0.8
Pineapple Peal	18.1	----	1.4
Potato Peel	2.2	----	----

1.1.3. Carbohydrates

Agro-Industrial residues are mainly composed of lignocellulose, which is the most abundant natural biomass in the world. Lignocellulose is mainly composed of cellulose, hemicellulose, and lignin. These constituents have shown potential as feedstocks to produce energy. [12]

Cellulose is the most abundant natural polysaccharide in the world, consisting of a linear chain of D-glucose monomers covalently linked by β-(1→4) glycosidic bonds (Figure 1.3). [12] Cellulose has two different forms of structure, the amorphous structure and the crystalline structure that is easier to break down into D-glucose monomers. [13]

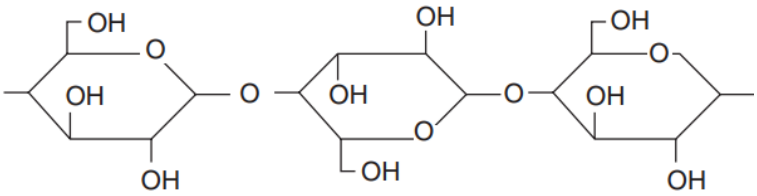

Figure 1.3: Structure of cellulose (Adapted from [13]).

Hemicellulose has an amorphous and random structure, being a heteropolysaccharide made of various sugar monomers such as xylose, arabinose, glucose, galactose, mannose, and rhamnose. Hemicellulose is more readily hydrolyzed to monomeric sugars during thermochemical pretreatment than crystalline cellulose. [12]

Lignin is a complex aromatic polymer, a phenyl-propanoic polyphenol with a non-crystalline and irregular three-dimensional structure. It is composed of coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol monomers (Figure 1.4). Figure 1.5 (A) is a schematic representation of the structure of lignocellulose. Lignin provides structural strength, and resistance against insects and pathogens and hemicellulose binds cellulose and lignin. [12], [13].

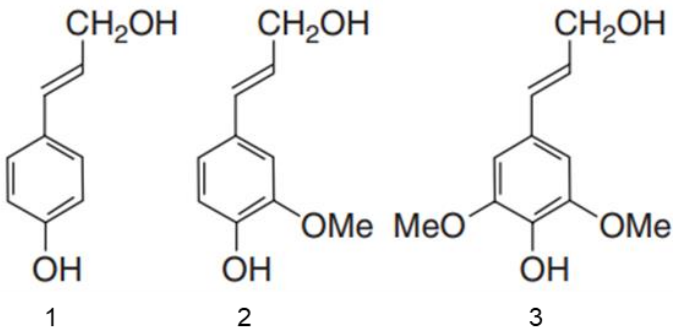

Figure 1.4: Lignin monomers: p-coumaryl alcohol (1), coniferyl alcohol (2) and sinapyl alcohol (3) (Adapted from [13]).

Agro-industrial residues usually have large quantities of glucose in the form of cellulose, which is a monomer that can be fermented by most microorganisms, originating a variety of products.

Since cellulose and hemicellulose are lignified to the cell wall, to break them into monomers its necessary to use a pre-treatment effective method. Figure 1.5 (B) is a schematic representation of lignocellulose after the pre-treatment.

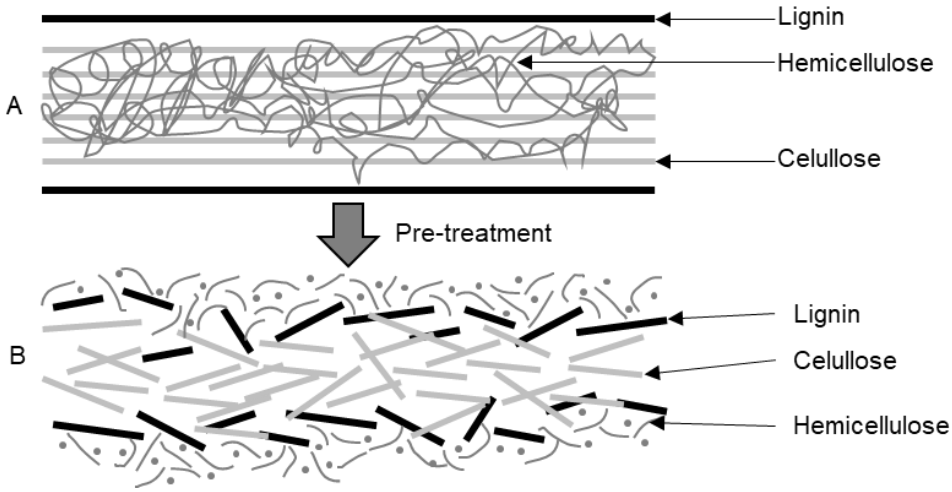

Figure 1.5: Schematic representation of lignocellulose: structure before pre-treatment (A) and after pre-treatment (B) (Adapted from [14]).

Pre-treatment process breaks down the lignin structure, allowing the enzymes or acids to access and hydrolyze cellulose. Even though it is ideally an economic step, pre-treatment methods can be the most expensive part of the process, having the potential to improve its efficiency, lowering the costs. There are several pre-treatment methods, such as physical, chemical, and biological, to separate cellulose and hemicellulose from the cell wall. Some of these methods are shown in Table 1.2. [13], [14]

Table 1.2: Pre-treatment methods to break down lignocellulose (Adapted from [14]).

Pre-treatment	Examples	Effect
Physical	Milling	Fine, highly de-crystalized structure
	Steaming treatment	Increased pore size/ hemicellulose hydrolysis
	Hydrothermal	Hemicellulose hydrolysis, alteration in properties of cellulose and lignin
	Irradiation	Depolymerization
Chemical	NaOH, NH_3, H_2O_2	Lignin/ hemicellulose degradation
	Peroxyformic acid	
	Organosolvents	
	Peroxymonosulphate	Activates delignification
Biological	White-rot fungi	Lignin degradation
	Specific Bacteria	
Enzymatic	Lignin Peroxidases	Selective lignin/ hemicellulose degradation

1.1.4. Phenolic Compounds

Phenolic compounds are the second most abundant group of organic compounds, after cellulose, in the plant kingdom. It is commonly known for its potent antioxidant activity, representing an essential role in potential health benefits. Phenolic compounds also participate in plant activities, such as structural support, protection against ultraviolet solar radiation, abiotic and biotic stress, and pathogens. They also have a role in fruits and vegetable attributes, such as color, flavor, and bitterness. It is generally distributed in plant tissues, including seeds, leaves, stems, fruits, roots, etc. [15] Beyond its antioxidant activity, some phenolic compounds have shown potential as antimicrobial and anticarcinogenic. [16] A healthy diet that includes a high intake of fruits and vegetables containing phenolic compounds is directly related to a reduced risk of developing cardiovascular diseases, obesity, diabetes, cancer, etc. Those health benefits are related to the presence of antioxidant phytochemicals that have a scavenging effect on reactive oxygen species, neutralizing their adverse effects. [15]

Phenolic compounds comprise an extensive range, from simple phenolic molecules to complex and highly polymerized compounds. These compounds consist of one or more aromatic rings connected with one or various hydroxyl groups. Phenolic compounds are classified according to the number of phenol rings and the components that bind these rings together. Phenolic compounds can be divided into five major classes: phenolic acids, stilbenes, lignans, flavonoids, and tannins. Phenolic acids and flavonoids together account for 90% of total dietary phenolic compounds. [17]

Phenolic acids are the most abundant phenolic compounds occurring in everyday dietary antioxidants in the population, accounting for 60% of total dietary phenolic compounds, and are mostly found in fruits. Phenolic acids can be benzoic or cinnamic acid derivates. P-hydroxybenzoic, vanillic, protocatechuic, gallic, and syringic acids are some of the most common hydroxybenzoic acids in fruits. These phenolic acids have a C6-C1 structure. Figure 1.6 (A) is a representation of some hydroxybenzoic acids. Some hydroxycinnamic acids are represented in Figure 1.6 (B) and include caffeic, p-coumaric, and ferulic acids. Hydroxycinnamic acids have a three carbon side chain (C6-C3), and are rarely found in their free form, usually bound to structural components of the plant cells or linked to small organic acids. [15], [17], [18]

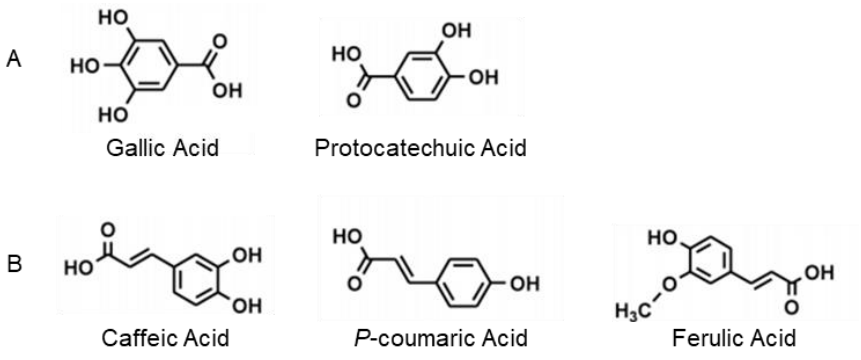

Figure 1.6: Representation of phenolic acids: hydroxybenzoic acids (A) and hydroxycinnamic acids (B) (Adapted from [19]).

Stilbenes are monomers or oligomers and have a 1,2-diphenylethylene core with hydroxyls substituted on the aromatic rings. Resveratrol is a naturally occurring phytoalexin produced in plants with anticancer properties, and its most common form is *trans*-resveratrol. It is found in grapes and berries. Figure 1.7 is a representation of resveratrol in its *trans* form. [17], [18]

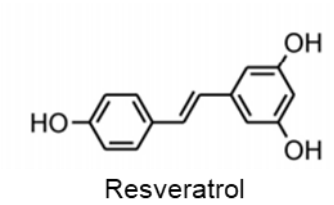

Figure 1.7: Representation of a stilbene: Resveratrol (Adapted from [19]).

Lignans comprise two phenylpropane units and can be found in small amounts in cereals, nuts, fruits, and vegetables, like pears and prunes. [15], [17]

Flavonoids account for 30% of total dietary phenolic compounds and are the most abundant phenolic compounds present in fruits. Its structure combines two aromatic rings bound by three carbon atoms, creating an oxygenated heterocycle. These can be divided according to the degree of oxidation into flavones, flavonols, isoflavones, flavanols, anthocyanins, flavanones, and proanthocyanins. These compounds occur as glycosides often, making them more soluble in water and react less toward free radicals. [17], [18]

Flavonols are usually present in high concentrations in fruits, like tomato, berries, citrus fruits, etc., and are typically associated with simple carbohydrates, such as glucose and rhamnose. Fruit usually contains 5 to 10 different flavonol glycosides primarily accumulated in the peel since sunlight stimulates their biosynthesis. Quercetin (Figure 1.8) is part of the flavonols group, and it is known for its anticarcinogenic and antioxidant properties. Flavones are less common in fruits and are usually glycosides of chrysin, luteolin, and apigenin. They are present in peels of citrus fruits and have free-radical scavenging and anti-inflammatory properties. Flavanones are typically found in tomatoes and citrus fruits, as aglycones like naringenin, hesperidin and eriodyctiol, and have anticancer properties. Flavanols are present in apricots and red wine, and can be either monomers, like catechin, or polymers, like proanthocyanins. Anthocyanins are present in berries and apples, and are responsible for the red, orange, purple, and blue colors. The most common glycoside is cyanidin, increasing as the fruit ripens. [17]

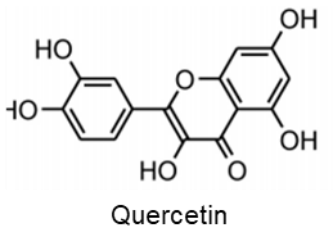

Figure 1.8: Representation of a flavonoid (Adapted from [19]).

Tannins are insoluble in water with molecular weights in the range of 500 to 3000. These are polymers, oligomers, or dimers of catechins linked between C4 and C8. They are present in apples, grapes, and pears. Its properties include antioxidant, anti-inflammatory, and chemical prevention of cancer. [17]

Phenolic compounds have many health benefits, such as reducing the risk of type 2 diabetes complications, anticarcinogenic, anti-aging, anti-inflammatory, antiproliferative, and antioxidant agents, and can also stimulate insulin secretion and attenuate hyperglycemia. These compounds are typically related to defense responses in the plant, as well as antifungal and antibacterial activities. Phenolic compounds are found in large quantities in food as natural antioxidants, and are even more abundant in fresh foods, like raspberries, apples, pears, cranberries, grapes, strawberries, etc. [18], [19]

Several methods to extract phenolic compounds from foods have been explored and developed in the latest years, alongside quantification methods to estimate total phenolic content. Quantification methods such as high-performance liquid chromatography (HPLC) and gas chromatography (G.C.) are more specific, identifying and quantifying individual phenolic classes. The most common quantification method is based on spectrophotometry. [18]

To quantify phenolic content, phenolic compounds should be extracted first, using a proper extraction method that will suit a quantification method. Various phenolic compound extraction methods include solid-liquid, ultrasound-assisted, microwave-assisted, supercritical fluid extraction, accelerated solvent, and high hydrostatic pressure. Some extraction methods are present in Table 1.3 [18]

Solid-liquid extraction is the most common and simple method to extract phenolic compounds and consists of a direct extraction with different solvents, such as methanol, acetone, ethanol, etc. A solid-phase extraction or chromatography column is necessary to remove unwanted substances. Ultrasound-assisted extractions are based on the influence of sound waves, ultrasonic wave frequency, and temperature to extract phenolic compounds at optimal conditions. Microwave-assisted extraction uses microwave energy to break analytes from the sample. Supercritical fluid extraction is an alternative extraction method that uses a supercritical fluid, usually carbon dioxide, based on the polarity of its sample, consuming less hazardous solvents. Accelerated solvent extraction extracts phenolic compounds under high pressure and temperature conditions, contributing to a fast solvent penetration in the sample cells avoiding phenolic compounds degradation. High hydrostatic pressure extraction is a high-pressure extraction method that stimulates a fast solvent penetration in the sample cells, causing an outflow of the cell components, such as phenolic compounds. [18]

Table 1.3: Phenolic compounds extraction methods (Adapted from [18]).

Extraction Method	Advantages	Disadvantages	Examples
Solid-liquid	Simple operation Great adaptability	Use of hazardous organic reagents Long extraction time Low efficiency	Catechins
Ultrasound-assisted	Simple operation Efficient Economical Great adaptability	Not specified for industrial production	Caffeic Acid Isoferulic Acid Ferulic Acid
Microwave-assisted	Less extraction time Consumes less extraction solvent Higher content of extracted antioxidants	Degradation and oxidation occur under such conditions	Caffeic Acid
Supercritical Fluid	Higher safety and selectivity Avoid sample oxidation	Unsuitable for polar phenolic compounds, unless a co-solvent is used High investment	Gallic Acid Protocatechuic Acid
Accelerated Solvent	Small amounts of solvent required Faster extraction	High temperature and pressure	Quercetin-3-O-glucuronide
High Hydrostatic Pressure	Efficient Small amounts of solvent required Less extraction time	High pressure Expensive equipment	Polyphenol

In this context, a different extraction method will be applied: subcritical water extraction. This method will be deepened further ahead.

1.1.1. Added-Value Residues

With increasing concern for environmentally friendly alternatives to reduce greenhouse gas emissions and pollution while adding value to agro-industrial residues and increasing profit, new technologies have been considered. Different studies have been done in that direction. Agro-industrial residues as feedstock in biorefineries have the potential to produce an extensive range of biobased products while contributing to sustainable development.

Agricultural industries produce about 3.7 billion tons of agro-industrial residues every year worldwide. Agro-industrial residues have about 40% cellulose, 30% hemicellulose, 20% lignin, 5% minerals and 5% proteins. Current applications for agro-industrial residues valorization include the production of antioxidants, flavonoids, phenols, carotenoids, lipids, phytochemicals, and as carbon sources. Cellulosic biomass has been used to produce biochemicals as

phytosterols, acrylic acid, polypropylene, thioester, isobutanol, and esters; and lignin to produce polyhydroxyalkanoates and adipic acid. [20]

Bio-based chemicals production is assessed to be around 50 million tons per year, yet most polymers and organic chemicals produced are oil and gas feedstocks derived. The petrochemical production of chemicals is around 330 million tons a year. Methanol, propylene, ethylene, butadiene, toluene, benzene, and xylene are used to produce plastics, polymers, and a range of other chemicals. Technically, most chemicals from fossil-based feedstocks can be substituted by bio-based chemicals. There are some conditions that prevent this ideal substitution from occurring, such as the bio-based production cost, which is higher than the petrochemical production, and the performance of new bio-based products should be equal or higher than the petrochemical products, as its environmental impact should be significantly lower, to make up for the producers' investment. [21]

The low crude oil price has been the barrier to bio-based chemical production growth. However, with the awareness for limited supplies and sustainability, consumer demand for clean and environmentally friendly products, and oil price rise, there is room for expansion. Bio-based products are now seen as a good and sustainable alternative to petrochemical-based products, worthy of investment and development towards that ideal. [21]

Many countries still depending greatly on fossil fuels imports, that will soon reach its pinnacle and the necessity to expand energy sources, due to climate change and to reduce greenhouse gases emission are the main drivers to transition to bio-based mass production. Bio-based products can be carried out in single processes, yet, to produce both secondary energy carrier and bio-based products, they can be integrated into biorefinery processes, in a more efficient approach. The transportation sector is also the main driver towards bio-based chemical production since all the transportation is made with fossil fuels. This is a major concern for this sector, facing the most recent policy regulations that impose that a large portion of the fuels utilized should be biofuel. Conventional (biodiesel, ethanol) and advanced (ethanol, butanol, lignocellulosic methanol) biofuels are not competitive with current oil prices. This condition avoids the entrance of biofuels into the market unless it is forced from the government or has financial support. [21]

Biofuel production costs can be reduced by co-producing added-value products and biofuels from biomass in an efficient approach, making the biofuel cost market competitive, depending on the biomass resource price. EIA created several different platforms considering the feedstocks, bio-based products, and the diversity of industrial processes. These platforms include a syngas platform, a biogas platform, a C6 and C5/C6 sugar platform, a plant-based oil platform, an algae oil platform, an organic solutions platform, a lignin platform, and a pyrolysis oil platform. Most platforms are still being studied. [21]

Synthesis gas (syngas), a mixture of carbon monoxide and hydrogen, can produce methanol, ethanol, ammonia, and potentially other chemicals and can be produced through the gasification of biomass. Biogas is produced through anaerobic digestion of high moisture content biomass,

giving origin to methane, which is used for its energetic value. Six carbon sugar platforms are produced through sucrose or hydrolysis of cellulose to give glucose, which is used as feedstock for biological fermentation, giving origin to several chemicals, or is converted by chemical processing. Mixed six or five carbon platforms are accessed through hydrolysis of hemicellulose, which, when fermented, can theoretically produce six-carbon sugar streams. Fermentation-derived chemicals, such as amino acids, enzymes, antibiotics, organic acids, etc., are an industry with over 20 years that produces around 8 million tons of products yearly. The major component of most plant oils, triacylglycerol, can be used to produce glycerol through transesterification. Under fermentation or anaerobic digestion, processes can produce epichlorohydrin and propylene glycol and other by-products formed in the process. Other platforms are still being studied and developed. [21]

Development of bio-based polymers and chemicals, where biomass replaces oil-based feedstocks in biorefinery complexes, will require more technology development and lead to new feedstock demand while making a significant contribution to sustainable development and adding value to various market sectors (agriculture, forestry, energy, and chemical). These technologies not only have great potential to replace obsolete processes but also add value to agro-industrial wastes, reduce their accumulation in the environment while creating more income for some sectors. [21]

1.1.2. Tomato waste

Tomato is a worldwide consumed fruit, with more than 182 million tons produced in 2018 (Figure 1.9). [22] There are about 7500 tomato varieties, with various sizes, shapes, and colors, going from the standard red tomato to yellow, pink, orange, purple, green, or white. Even though the largest producer is China, European countries present higher productivity in the tomato production industry. [23] Asia alone was responsible for 60.5% of tomato production in 2012, with 50 million tons, followed by the Americas with 15.3% and Europe with 12.8%. Oceania is the smallest continent producing tomatoes, with only 0.3%. Portugal produced 1.4 million tons of tomatoes that year, about 0.9% of the world production, becoming the 15th world producer. [24]

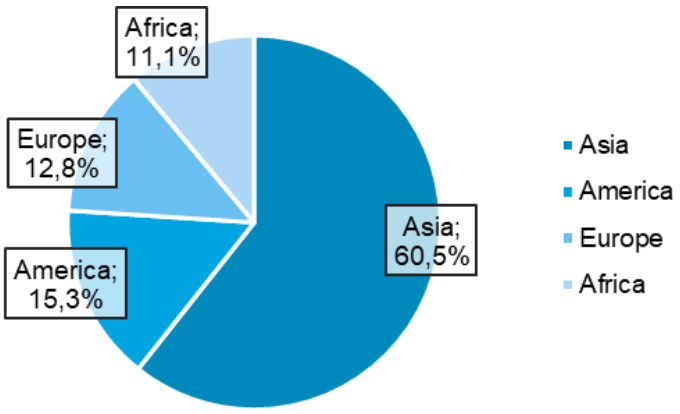

Figure 1.9: World tomato production by continent (Adapted from [24]).

Tomato is highly consumed in the form of ketchup, tomato juice, purée, and sauce. During the processing of tomato, tomato waste, or pomace, is created, a tomato by-product, representing about 4% of the fruit weight. [25] This waste is usually underused and redirected to animal feed or compost. However, various studies have shown that tomato by-products can be used to create new products at low costs, such as vitamins, pectin, essential oils, and carotenoids. [26] Lycopene is a carotenoid abundantly found in tomato. It is responsible for the red color of tomatoes, is insoluble in water and soluble in fat, and can differ between 0.88 to 4.2 mg/ 100 g in fresh tomatoes. Lycopene bioavailability increases with tomato processing, as ketchup, for example, contains 9.9 to 13.44 mg of lycopene for 100 g. Its antioxidant properties are responsible for disease prevention, such as prostate cancer. [27]

Tomato waste is mainly composed of peel, pulp, seeds and can also contain some leaves and small branches of the plant, depending on its origin. Tomato waste composition has been studied over the years. Its primary component is fiber, around 51-25%, followed by protein, about 23-15% dry weight. This waste is rich in carbohydrates in the form of cellulose, hemicellulose, lignin, and fibers. When being characterized, total lipid content, total phenolic content, and ash content are also determined. Phenolic content is greater in tomato peel, in the form of polyphenols and ascorbic acid, while lycopene has 2.5 times higher levels in the peel than the pulp. [25], [28]

It has been identified in former studies that tomato contains a range of phenolic compounds, granting its antioxidant properties, contributing to a healthier diet. This content varies greatly depending on the part of the fruit taking into consideration. Tomato peel contains a higher amount of these compounds than pulp and seeds. Phenolic compounds identified in tomato waste vary in each study due to differences in the several fruit parts. Phenolic compounds identified include caffeic acid in its simple and conjugated forms, being the most abundant phenolic acid, p-coumaric, chlorogenic acid, ferulic acid, gallic acid quercetin, catechin, naringenin, as major flavonoid, rosmarinic acid, and rutin. [28], [29]

Tomato is rich in several nutrients, such as vitamin B and C, potassium, phosphorus, magnesium, dietary fiber, calcium, etc. Tomato waste research is gaining more interest due to many health benefits, like cancer prevention, fertility improvement, avoid cardiovascular diseases and old-age-related diseases, like osteoporosis, dementia, Parkinson’s, and Alzheimer’s disease. Tomato waste applications are yet being studied, having great potential in the health field. [24]

Tomato contains a range of phenolic compounds, contributing to a healthy diet. Phenolic compounds' content varies between the pulp, and the peel, which includes a higher amount of these compounds. [28] A large amount of tomato waste from industrial processing is produced and addressed mainly for animal feeding or compost. This waste can be recovered through chemical and biochemical processes to produce high-value metabolites, being an excellent alternative to obtain natural antioxidants. Therefore, there is great interest in exploiting the tomato waste of industrial processing to recover natural phenolic compounds. [29]

The aim of this work is the valorization of tomato waste from the tomato processing industries. It has great potential as raw material for biorefineries, since it is rich in carbohydrates, lignin, and bioactive compounds, such as antioxidants. Pharmaceutical, chemical, and food sectors are some possible applications for this agro-industrial residue. [25]

1.2. Subcritical Water Extraction

As discussed previously, extraction is an important step in agro-industrial residues valorization. Extraction consists of a chemical mass transfer from one phase to another and is present in the simplest actions, such as making coffee or juice. [30] Chemically speaking, extraction processes offer the opportunity to explore new products applications for the pharmaceutical, environmental, biotechnological, food, and chemical sectors. Hazardous solvents for extraction processes have been a major issue due to recent policies and awareness for cleaner technologies, greenhouse gas emission, and waste accumulation issues. For that reason, environmentally friendly processes have been getting more attention. [31] To improve extraction procedures and reduce organic solvents in this processes, new technologies are being introduced, such as microwave assisted extraction, accelerated solvent extraction, pressurized liquid extraction, supercritical fluid extraction and subcritical water extraction (ScW). ScW extraction is also named superheated water extraction or pressurized hot water extraction. [30]

1.2.1. Water properties

Water is the foundation of life, the major component of all living things. All living beings require water for their survival, while it is one of the most basic molecules know. Its unique properties have engaged many scientists' attention; however, many of its physical properties have no explanation yet. [32]

Like many substances, water has three physical states, solid, liquid, and gas. It is composed of two hydrogen and one oxygen atom, making it a highly polar solvent at room temperature and atmospheric pressure. Due to its extensive hydrogen bonding structure, water has a high dielectric constant. For this reason, water was not considered as an efficient extraction solvent to extract organic or non-polar compounds. Some of the water physical properties can be found in Table 1.4. [33]

Table 1.4: Some physical properties of water at 25ºC (Adapted from [32]).

Physical Property	Water
Formula	H_2O
Molecular Weight (g/mol)	18
Density (Kg/L)	0.998
Fusion point (ºC)	0
Boiling point (ºC)	100
Specific heat (J/ K.g)	4.18
Vaporization Heat (kJ/g)	2.3
Surface tension (mN/m)	72.8
Viscosity (µPA s)	1002
Dielectric constant	78.6

When water surpasses its boiling temperature and pressure is increased (Figure 1.10 B), hydrogen bonds of the molecule break, changing its properties. Water physical behavior under different temperature and pressure conditions is described in Figure 1.10. [33]

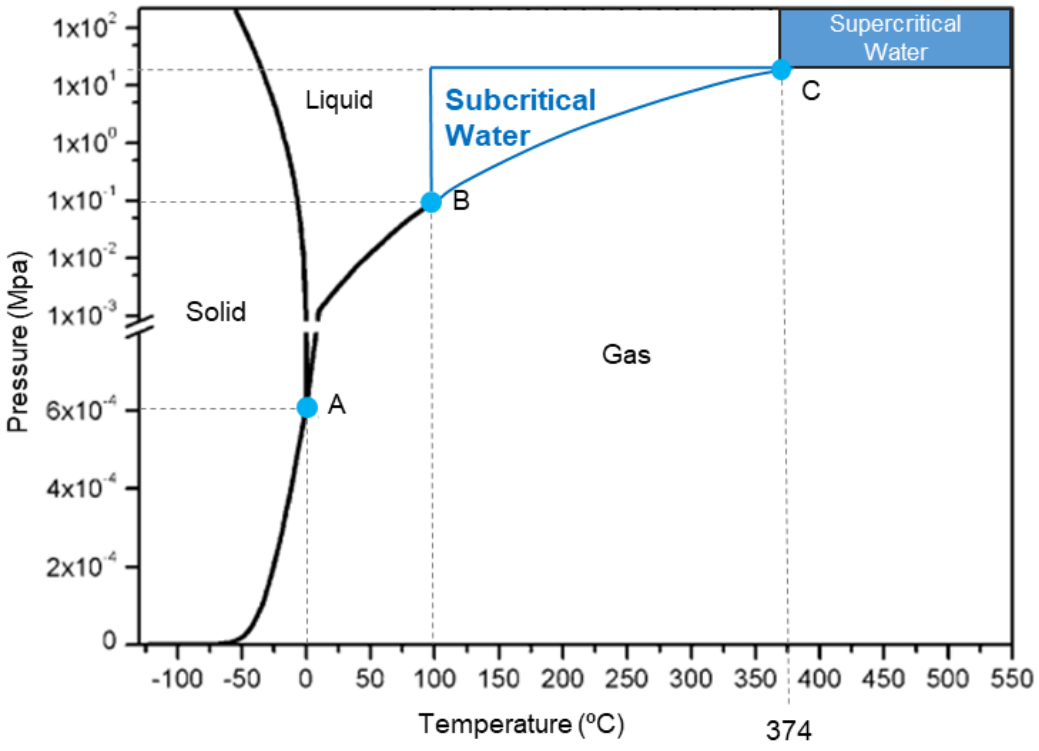

Figure 1.10: Water phase diagram: triple point (A), boiling point (B), and critical point (C) (Adapted from [34]).

Dielectric constant, viscosity, and surface tension of water decrease with the increase of temperature. When at high temperatures, water can be kept at its liquid state by increasing the pressure. Under these conditions, water properties are similar to organic solvents, being able to dissolve several low and medium polarity compounds. By changing these parameters, properties such as the dielectric constant can be manipulated. [33]

1.2.2. Subcritical Water

ScW extraction is distinct from other technologies for its efficiency, safety, selectivity, lower price, faster and for being environmentally friendly. It is hot water at sufficient pressure to maintain its liquid state above its boiling temperature (100 ºC) and below its critical point (374 ºC) and under the critical pressure (22.1 MPa). [33]

ScW extraction allows such manipulation of extraction conditions that it is possible to extract different classes of compounds with different polarities by changing the temperature, with more polar extracted at low temperature and less polar at high temperatures. [35] At 20 MPa and 300 ºC the dielectric constant of water is approximately 20, which allows the extraction of non-polar compounds, like highly complex molecules. This would be impossible to achieve at atmospheric pressure. [33]

For a good ScW extraction, four consecutive steps should be followed. First, the solute is desorbed at several active sites in the matrix under high temperature and pressurized conditions. Then, the diffusion of the extracts into the matrix occurs. Next, the solutes can split themselves from the matrix into the extraction fluid, and finally, the sample solution is eluted and collected from the extraction cell by chromatography. As temperature of ScW increases, so does the ability to dissolve analytes and the penetrability of the matrix particles (diffusivity). [33]

Several factors can influence ScW extraction, such as temperature, pressure, solvent flow rate, particle size, extraction time, etc. Temperature is the most crucial factor, having the ability to affect extraction efficiency and selectivity. Extraction temperature must be suitable according to the compound being extracted. As the temperature increases, water goes from polar to non-polar, promoting the dissolution of less polar compounds in water. However, high temperatures can cause the degradations of compounds and contribute to oxidation and hydrolysis reactions. Unlike temperature, pressure has no significant effect on the recovery efficiency, and it is mainly controlled to make sure that ScW is kept in the liquid state. A high flow rate can improve the extraction rate of compounds, and consequently, extraction efficiency. Particle size is a determinant factor of ScW extraction since a small particle size implies a high extraction yield. Smaller particles reduce the diffusion distance of compounds in the matrix, increasing the contact area of the matrix and the extractant, reducing extraction time. [36]

Several studies have been made using ScW extraction in diverse sectors, such as biotechnology, biochemistry, food, pharmaceutical, etc., and it is a good alternative to current extraction methods for its efficiency, selectivity and for being a cleaner technology. Applications for ScW extraction

are still being studied; however, the extraction of polyphenols or antioxidants from black tea, rosemary,[37] *Coriandrum sativum* seeds [35], steviol glycosides from *Stevia rebaudiana* leaves [38], volatile fatty acids from food waste [39], extracts (catechol, gallic acid, pyrogallol, etc.) from watermelon [40], flavonoids form ginseng leaf/stem [41], hemicellulose from wood [35], bioactive compounds from benne hull [42], sardine [43], grape pomace [44], [45] and spent coffee grounds [46], seed oil from *Nitraria tangutorum* [36], etc. are some of the many studies already carried out successfully. ScW extraction also has excellent potential in the valorization of agro-industrial residues.

1.3. Thesis Outline

With an increase of energy demand, renewable and clean technologies are getting more attention, to reduce greenhouse gases emissions and pollution. Biorefineries can use agro-industrial residues to produce added-value residues, thus adding value to previously not valuable wastes. Agro-industrial residues are rich in carbohydrates, phenolic compounds, as well as other bioactive compounds and may have a great range of applications in several sectors. Carbohydrates and phenolic compounds can be extracted using various technologies for further valorization. Subcritical water extraction is a clean and environmentally friendly technology, that can be used to efficiently extract added-value compounds, while avoiding the use of conventional organic solvent extractions.

Tomato waste is a by-product of the tomato processing industries. This is an agro-industrial residue with great potential in several sectors, such as pharmaceutical, food, biochemical and biotechnology. The main goal of this work is to assess the potential valorization of tomato waste, using subcritical water to extract/hydrolyze valuable compounds that can posteriorly be applied in a diverse range of sectors. For this matter, tomato waste was chemically characterized to evaluate its potential as an added-value residue. Carbohydrate content, which can be used to produce energy, for example, and phenolic compounds were target compounds expected to find in this residue. For a greater efficient recovery of these compounds, tomato waste was submitted to ScW extraction, where these compounds would be extracted at high temperatures, with a higher yield and biomass conversion than methods previously described. Antioxidant activity of the extracts was studied, as another application of the tomato waste residue.

Chapter 2

Materials and Methods

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2.1. Materials

2.1.1. Tomato Waste and Tomato Waste Powder

Tomato Waste (TW) was provided by the Portuguese company, HIT tomato group, consisting of seeds, pulp, and peels, of the tomato processing. Tomato Waste was received fresh, frozen with liquid nitrogen, and lyophilized (lyophilizer CHRIST ALPHA 1-4, Braun Biotec International) for 3 days. Then, it was milled in a grinder to tomato waste powder (TW powder) and stored in the dark, inside plastic bags under a nitrogen atmosphere at -18ºC.

2N Folin-Ciocalteau's reagent, phenol (99%), gallic acid monohydrate (98%), D (+)-glucose monohydrate 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich. All other reagents and solvents used in this study were of analytical grade and purchased from available suppliers.

2.2. Chemical Characterization of Tomato Waste

2.2.1. Water Content

The water content of TW powder was determined with a moisture analyzer (KERN DAB 200-2). This analysis was performed in triplicates.

2.2.2. Protein Determination

Protein content in biomass samples is hard to measure directly. Nitrogen content was determined by elementary analysis (CHNS technique) of 0.3 g of the TW powder. Therefore, a nitrogen factor of 6.25 was used, as recommended in previous methods. Elementary analysis was performed at Laboratório de Análises, REQUIMTE-LAQV at FCT/UNL. [47]

2.2.3. Ash Determination

Inorganic material in TW powder was determined as ash content. Ash content was determined by measuring 0,8 g of TW powder in a porcelain crucible and placing them in a muffle furnace at 550 ºC for 6 hours. After this time, the porcelain crucible was removed and placed in a desiccator to cool down. The inorganic material was weighted, and ash content was determined through mass difference. [48]

2.2.4. Lipid Content

For lipid content determination, approximately 2 g of TW powder were weighted in a filter paper packet, posteriorly placed in a Soxhlet apparatus. The extraction occurred using 70 mL of n-hexane under heating for 3 hours. After the extraction, a flask was weighted, and the remaining n-hexane and extracted oil were transferred to that flask. N-hexane was evaporated using nitrogen, and lipid content was obtained by subtracting the flask mass to the final weight. [44]

The defatted residue in the filter paper packet was dried overnight at 40 ºC and weighted afterward.

2.2.5. Carbohydrates and Lignin Content

Carbohydrates and lignin usually are the major portion of a biomass sample; therefore, they must be measured to take part in a complete characterization. Carbohydrates are either structural or non-structural (free). The latter represent soluble carbohydrates and can be removed through extraction and washing steps. The first one is bound to the matrix of biomass, being insoluble therefore requiring a more complex extraction. Lignin is a complex phenolic polymer. [49]

About 0.8 g of defatted TW powder was extracted with 40 mL of ethanol: water solution (80:20 v/v) in an ultrasonic bath for 15 min at room temperature. This procedure was performed in duplicates to make sure they were concordant. The mass of both recipients was equated using the same solution. The recipients were placed in a centrifuge for 10 minutes, at 4 ºC and 10 000 rpm (Beckman Coulter Avanti J-26 XPI centrifuge, Rotor JA-25). Next, the supernatant was removed to a flask, and a new solution was added. This process was repeated three times, and the three supernatants were combined. Ethanol was evaporated at 50 ºC, under vacuum, in a rotary evaporator (Steroglass Strike 300 Rotary Evaporator). To the remaining solution, water was added up to 100 mL for dilution and used for carbohydrate quantification. [49]

The defatted TW powder, without soluble carbohydrates, left after removing the supernatants was dried at 40 ºC overnight and used for the next step, the hydrolyzation of the insoluble carbohydrates. In this step, 0,3 g of the residue was added to a flask, and 3 ml of 72% (w/w) H_2SO_4 were added. This mixture was placed in a water bath at 30 ºC, for 1 hour, under stirring. Following, the acid was diluted to 4% (w/w) H_2SO_4, adding 84 mL of water, and placed in a silicone bath, for another hour at 121 ºC, under stirring. After cooling to room temperature and filtered, the supernatant was used for insoluble carbohydrate quantification. The solid was washed with water, dried at 105 ºC and weighted, and corresponds to lignin. [50]

Both soluble and insoluble carbohydrate solutions were used for carbohydrate HPLC analysis.

2.2.6. Phenolic Compounds

For phenolic compounds extraction, 1 g of TW powder was placed into a flask, and 40 mL of a (60:40 v/v) methanol: water solution was added. The mixture was incubated for 90 minutes, 60ºC, under stirring. Following, the mixture was filtered, and the solution was used for phenolic quantification.

2.3. Quantification Methods

2.3.1. Total Carbohydrate Quantification

This method allows to quantify the sugar content of the previous solutions. For total carbohydrate quantification, a calibration curve with D (+)-glucose monohydrate was built from a 1 g/L stock

solution, diluting it to 0.1 g/L, 0.075 g/L, 0.05 g/L, 0.025 g/L, 0.01 g/L and 0.005 g/L solutions in distilled water, using only distilled water for the blank solution. The calibration curve was successfully determined with a R^2 above 0.99 (Chapter 7: Appendix, Figure 7.1) Sugar-rich solutions used for this method were submitted to a solid precipitation process in a centrifuge for 15 minutes at 12 000 rpm (Heraeus Sepatech, Biofuge 13 Centrifuge) and diluted when needed.

To 500 μL of either the standard solutions or an experimental solution (sugar-rich solution), 1.5 mL of H_2SO_4 (96%) and 300 μL of a 5% (w/v) aqueous phenol solution were added. The mixtures were stirred and incubated for 5 minutes in a Accu BlockTM Digital Dry Bath, at 90 ºC. Following, mixtures were stirred and cooled to room temperature in a water bath. The absorbance of each sample was measured at 490 nm in a Thermo Scientific GENESYS 50 spectrophotometer and the results were expressed in g/L glucose equivalent (g GE/L). [50]

2.3.2. Total Phenolic Quantification – Folin-Ciocalteau Method

Total phenolic content was determined using the Folin-Ciocalteau Method. A stock solution of 5 g/L of gallic acid monohydrate (98%) was used to build a calibration curve with 1 g/L, 0.75 g/L, 0.5 g/L, 0.25 g/L, 0.1 g/L, 0.05 g/L, and 0.025 g/L standard solutions in distilled water. The blank was distilled water. The calibration curve was successfully determined with a R^2 above 0.99 (Chapter 7: Appendix, Figure 7.2) A protein precipitation step was needed before applying this method, so that these would not interfere with phenolics quantification. For that, to 800 μL of the experimental solution, 120 μL of 100% (w/v) trichloroacetic acid were added, the mixture was well stirred and stored at -20 ºC for 5 minutes. After that, the solution was stored for 15 minutes at 4 ºC and centrifuged for 15 minutes at 12 000 rpm.

To 20 μL of either the standard solutions or the experimental solution, 1.58 mL of distilled water and 100 μL of the Folin-Ciocalteau reagent were added. The mixture was stirred and left at room temperature for 5 minutes. Then, 300 μL of a sodium carbonate solution was added, the mixture was stirred and incubated for 30 minutes at 40 ºC in a Accu BlockTM Digital Dry Bath and stirred again at the end of that time. Absorbance was measured at 750 nm in a Thermo Scientific GENESYS 50 spectrophotometer, and the results were expressed in g/L gallic acid equivalent (g GAE/L). [44]

2.3.3. HPLC method for carbohydrate analysis

HPLC was used to quantify a small range of standard carbohydrates, such as glucose, fructose, galactose, arabinose, mannose, sucrose, and fucose in ScW extracts and soluble and insoluble carbohydrate solutions. These analyses were performed at Laboratório de Análises, LAQV-REQUIMTE, using a Dionex ICS-3000 system with electrochemical detection. This was performed at 40ºC using a 4x50 Thermo BioLC Dionex AminoTrap pr-column and a 5x250 mm Thermo Dionex CarbonPac SA10 column.

Calibration curves were built at 25, 50, 75, 100, 150, and 250 ppm for the monosaccharides, using 4% (w/w) sulfuric acid solution, since there is a destruction of sugars during acid hydrolysis. A 1 mM NaOH solution was used as mobile phase and kept at a 1.2 mL/min flow rate. [49]

2.4. Subcritical Water Extraction

2.4.1. ScW extraction unit

The subcritical water extraction unit includes a high-pressure pump (KNAUER 40 Preparative pump 1800), connected to a distilled water deposit, that pumps water through a high-pressure tube to a stainless-steel high-pressure reactor, with 51 cm long, 5 cm external diameter and 2.6 cm internal diameter. The pumped water was heated by a heating wire connected to a temperature controller before reaching the reactor. The reactor was placed inside an electric oven with temperature control. Temperature regulators were placed before and after the oven. Pressure was measured with a pressure indicator at the oven's exit and controlled by a Back-Pressure Regulator (BPR; Tescom Europe®, 26-1000). The aqueous effluent from the reactor passed through a filter and was collected at the end of the process for further analysis. Figure 2.1 is a simplified representation of the experimental setup (TI and PI are temperature and pressure indicators, respectively) and Figure 2.2 is the apparatus used for hydrolysis and extraction with ScW.

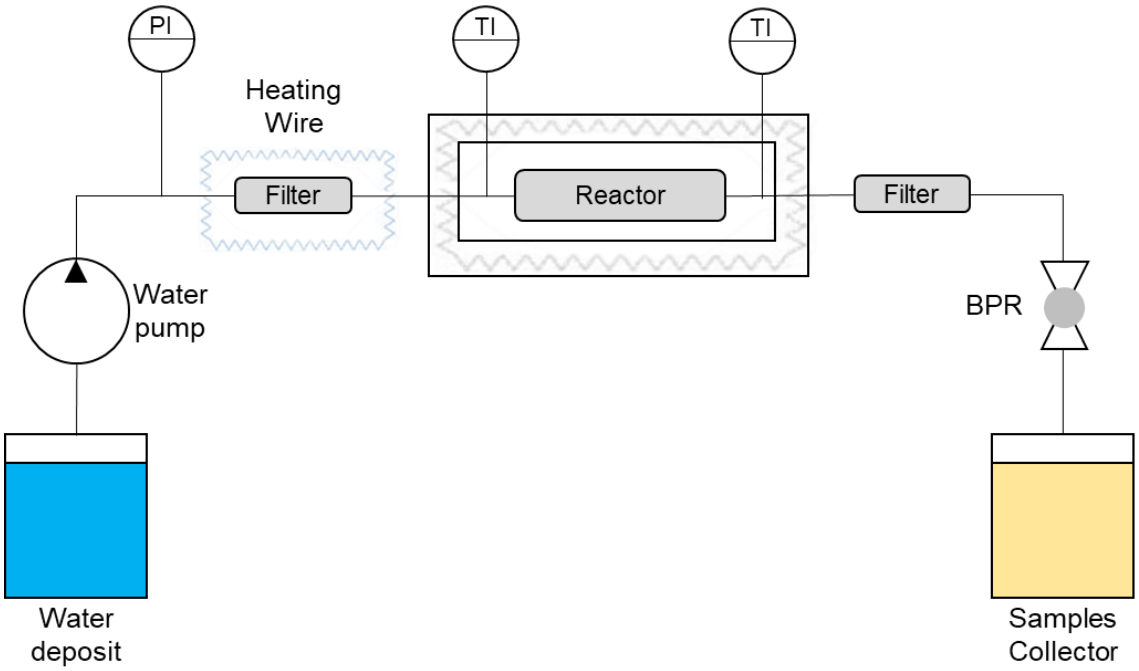

Figure 2.1: Simplified schematic representation of the ScW experimental setup.

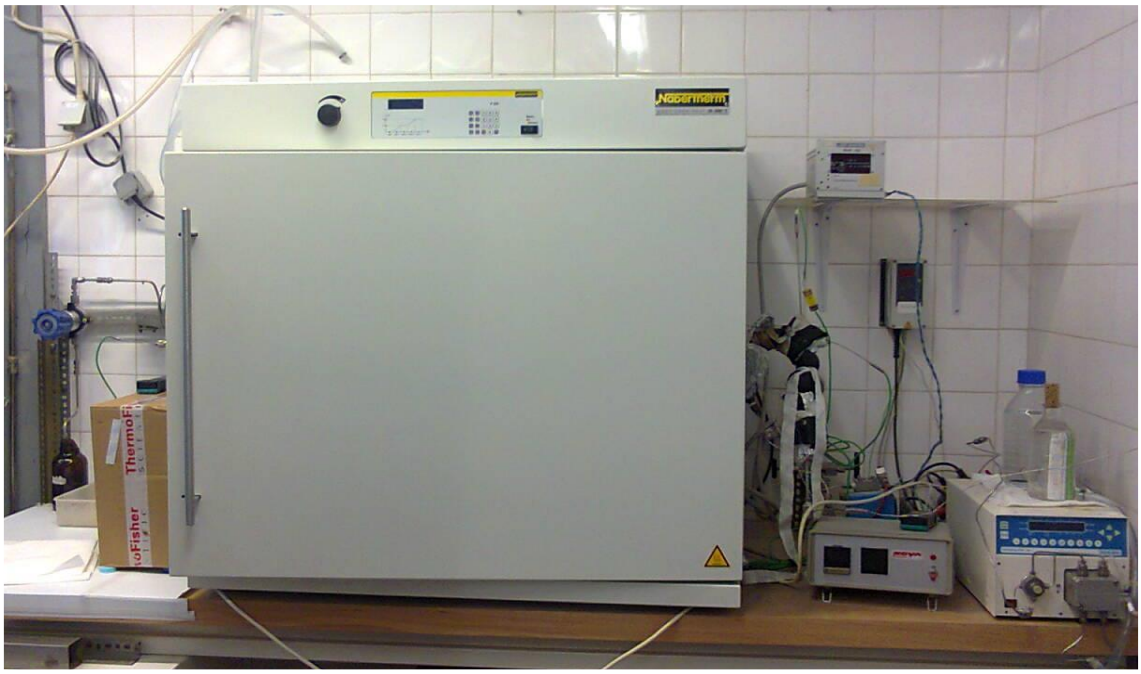

Figure 2.2: ScW apparatus used for hydrolysis and extraction of DTW powder.

In an experiment, the reactor was filled with ca. 18.5 g of DTW powder, placed between porous discs. The pressure was kept constant at 100 bar during the experiment. A constant water flow of 12 mL/min was used. Once it took a certain time to reach the desired temperature in the reactor, the liquor leaving the reactor was collected in a series of sampling tubes, and seven samples were collected. The first sample was collected until the water reached 50 ºC (ramp), and the second one, from 50 to 140 ºC (ramp). Once the water temperature reached 140 ºC, the temperature was kept constant for 30 min, while collecting the third sample (stage). The same procedure was made for the following target temperatures of 190 ºC and 250 ºC, by collecting samples in between them and another for 30 min at those temperatures.

2.4.2. Extraction Efficiency and Extracts Characterization

Sugar-rich collected samples, herewith called ScW extracts, were used for quantification based on quantification methods previously described. ScW extracts were stored at -20 ºC.

For each extract, 10 mL were lyophilized to calculate the ScW extract yield. After the quantification methods were applied to all seven extracts, the first three extracts were mixed, giving the 140 ºC extract, doing the same for the 190 ºC and 250 ºC extracts. These were lyophilized and used for chemical characterization as described previously, including HPLC analysis.

The reactor residue was dried at 105 ºC until it was completely dry and weighted. By comparing the final mass of the residue with the initial DTW powder mass placed inside the reactor, it was possible to obtain the extent of extraction/hydrolysis of DTW. The reactor residue was also chemically characterized for protein, ash, carbohydrate, and lignin content.

2.5. ScW Extracts Activity

All methods described in this section were performed after solid phase extraction.

2.5.1. Solid Phase Extraction

For this assay, TW extract solutions were filtered to make sure that any precipitate was removed. Each column was neutralized with 4 mL of methanol and 4 mL of a 1% HCl solution. Then, 50 mL of each solution was applied in SPE columns (Strata™ X C-18 columns from Phenomenex), to extract polar organic analytes such as phenolic compounds. The 50 mL TW sugar-rich extracts were stored for posterior utilization. The analytes retained in the SPE column were eluted with 5 mL of methanol, bringing the concentration of these compounds ten times higher than their concentration in the original extract. These solutions were used for GC-MS characterization and for the following colorimetric tests. These solutions were diluted for this assay in a 1:5, 1:10, and 1:15 proportion in methanol.

2.5.2. Total Flavonoid Content

Flavonoids are a large group of polyphenolic secondary metabolites that can be found in plants, fruits, and flowers. Total flavonoid content was determined spectrophotometrically (Biochrom Libra S4) based on the formation of a flavonoid-aluminum complex. A chatequin solution (1 g/L) was used to build a calibration curve with 200 mg/L, 100 mg/L, 80 mg/L, 60 mg/L, 40 mg/L, 20 mg/L, 10 mg/L and 0 mg/L standard solutions in distilled water, in duplicates. The calibration curve was successfully determined with a R^2 above 0.99 (Chapter 7: Appendix, Figure 7.4)

To 500 μL of both standard solutions and TW extract diluted solutions (1:5), 2 mL of distilled water and 150 μL of a $NaNO_2$ solution (5%) were added. The mixture was allowed to stand for 6 minutes. After this time, 150 μL of an $AlCl_3$ solution (10%) were added and allowed to stand for 6 more minutes. After, 2 mL of a NaOH solution (4%) were added, and 200 μL of distilled water, to bring the final volume to 5 mL. The mixture was stirred and allowed to stand for 15 minutes. As some precipitate had been formed using this method, TW extract-based mixtures were filtered. The intensity of pink color was measured at 510 nm, and the results were expressed in mg of chatequin equivalents per g of extract. [51]

2.5.3. Total Phenolic Content

Folin-Ciocalteau method was used to quantify phenolic compounds after their isolation using SPE columns since some studies consider that there is an interference between sugar-rich extracts and total phenolic content. [52]

The total phenolic content of the extracts was determined spectrophotometrically (Biochrom Libra S4) using the Folin-Ciocalteau method. A calibration curve was built with gallic acid as a standard with 200 mg/L, 100 mg/L, 80 mg/L, 60 mg/L, 40 mg/L, 20 mg/L, 10 mg/L, and 0 mg/L standard

solutions in distilled water, in duplicates. The calibration curve was successfully determined with a R^2 above 0.99 (Chapter 7: Appendix, Figure 7.3)

To 500 μL of both standard solutions and TW extract diluted solutions (1:10), 2 mL of distilled water and 500 μL of Folin-Ciocalteau reagent were added and allowed to stand for 5 minutes. After this time, 2 mL of sodium bicarbonate (10% m/V) were added, and the mixture was incubated in the dark for 1 hour. Absorbance was measured at 760 nm, and the results were expressed in mg of gallic acid equivalents per g of extract. [53]

2.5.4. Antioxidant Activity with DPPH

2,2-diphenyl-1-picrylhydrazyl, (DPPH) is a dark purple colored powder, a stable radical, that becomes colorless or pale yellow when it is neutralized. This assay was based on measuring the loss of the purple color from DPPH after reacting with ScW extracts.

DPPH solution was prepared at 45 mg/L in methanol and stored in the dark. This solution could be used for one day. For this assay, the 1:10 extract solutions were used.

A 1 g/L Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) solution was used to build a calibration curve with 200 mg/L, 100 mg/L, 80 mg/L, 60 mg/L, 40 mg/L, 20 mg/L, and 0 mg/L standard solutions in methanol, in duplicates. The calibration curve was successfully determined with a R^2 above 0.99 (Chapter 7: Appendix, Figure 7.5)

To 500 μL of both standard solutions and diluted solutions, 4 mL of the DPPH solution were added, and incubated at room temperature for 30 min, in the dark. After that time, absorbance was measured at 517 nm, and the results were expressed in mg of Trolox equivalents per g of extract using the calibration curve obtained. This assay was based on measuring the loss of the purple color from DPPH after reacting with ScW extracts. [54] Therefore, to evaluate the free radical inhibition, Equation 2.1 was used.

$$\%\ inhibition = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \qquad \text{Equation 2.1}$$

In this equation, A_{blank} is the absorbance of the blank sample and A_{sample} is the absorbance of each sample containing the extracts.

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Chapter 3

Results and Discussion

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3.1. Chemical Characterization of Tomato Waste

Tomato Waste residue was provided by a Portuguese company, consisting of seeds and peels of processed tomato. The residue was lyophilized and grinned to TW powder. Some of the TW powder was submitted to supercritical CO_2 extraction to remove most of its lipid content and most of the lycopene. The extract thus obtained, rich in lipids and lycopene, was sent back to the company for further utilization. The residue that was left from the supercritical extraction was collected and labelled defatted TW powder (DTW powder). This residue was further used for ScW extractions.

Both TW powder and DTW powder were chemically characterized for water, protein, ash, lipid, structural and free carbohydrates, lignin, and total phenolic content. ScW extracts were characterized for protein, phenolic, flavonoid, and free carbohydrate content. Antioxidant activity was also evaluated with the DPPH assay. ScW residue was characterized for protein, ash, structural carbohydrates, and lignin content.

3.1.1. TW Powder and DTW Powder Characterization

The main components identified in both tomato waste residues, TW powder and DTW powder, are described in Table 3.1. Their composition was determined using the methodologies described in chapter 2.2 and chapter 2.3. These components are protein, ash, lipids, lignin, structural and non-structural carbohydrates, and phenolic compounds. Water content was also determined as being above 7%.

Table 3.1: Composition of TW and DTW powder (NS = Non-structural; S = Structural).

Component	g $_{component}$ /100 g $_{powder}$	
	TW powder	DTW powder
Protein	15.21 ± 1.50	17.85 ± 4.30
Ash	5.18 ± 0.07	5.09 ± 0.01
Lipids	4.99 ± 0.07	1.68 ± 0.20
NS. Carbohydrates*	19.85 ± 0.71	15.07 ± 0.92
S. Carbohydrates*	18.22 ± 0.76	15.43 ± 0.51
Lignin	19.22 ± 0.53	19.08 ± 0.18
Phenolic Compounds**	0.57 ± 0.03	0.58 ± 0.03
Water	7.10 ± 0.29	7.71 ± 0.17
Total	90.3 ± 4.0	82.5 ± 6.3

*Units in g GE /100 g $_{powder}$; ** Units in g GAE /100 g $_{powder}$

In both residues, the main component is carbohydrates, as expected since most fruits usually have a high content of sugars [55]. Carbohydrate content was quantified using the phenol-sulfuric carbohydrate quantification method. This is a simple colorimetric method that detects all classes of carbohydrates, from monosaccharides to polysaccharides, and its results are express in glucose equivalents. Both structural and non-structural carbohydrates are part of the cellulose and hemicellulose structure. For carbohydrate quantification, structural and non-structural sugars

were separately evaluated. In both residues, non-structural carbohydrates and structural carbohydrates values are similar, and above 18% in TW powder. TW powder is a heterogeneous residue, and each sample taken will have small variations in composition, that can be an explanation for total carbohydrate difference in TW and DTW powder.

Carbohydrate monomers were identified by HPLC, present in Table 3.2. The most abundant carbohydrate in both residues is glucose, since cellulose is composed of glucose, and hemicellulose has several sugar monomers, including glucose. [12] The second most abundant carbohydrate is fructose, present, mostly, in its non-structural form.

Table 3.2: Identification and quantification of carbohydrates in TW and DTW powder using HPLC.

Carbohydrate type	Sugar	$g_{sugar}/100\ g_{powder}$	
		TW powder	DTW powder
Structural Carbohydrates	Glucose	4.36	5.43
	Fucose	0.03	0.04
	Rhamnose	----	0.12
	Arabinose	0.82	0.88
	Galactose	1.19	1.12
	Xylose	2.33	2.51
	Fructose	----	1.36
	Total	8.7	11.5
Non-structural Carbohydrates	Glucose	8.37	5.80
	Rhamnose	0.01	----
	Arabinose	0.02	0.04
	Galactose	0.06	0.05
	Fructose	8.69	6.63
	Total	17.2	12.5
	Total	**25.9**	**24.0**

When comparing carbohydrates quantities using HPLC and carbohydrate quantification methods, there is a noticeable difference. First, a colorimetric quantification method, as referred earlier, identifies all carbohydrates, and not only monomeric sugars, while HPLC analysis only identifies certain sugar monomers. Second, not all sugars were identified in HPLC. Sugars such mannose, galacturonic acid, glucuronic acid, etc., were not taken into consideration when applying the HPLC method, which can be another explanation the difference between both methods. Table 3.3 presents carbohydrate identification and quantification in a similar report., in which simple sugar monomers were identified, as well as some polysaccharides. Sugar quantification values in this report are in the same order of magnitude, and concordant with results obtained using HPLC analysis.

Table 3.3: Carbohydrate identification and quantification in a similar report (Adapted from [56]).

Sugar	g $_{sugar}$ /100 g $_{TW\ powder}$
Glucose	6.65
Rhamnose	0.32
Arabinose	0.65
Galactose	0.65
Xylose	0.25
Fructose	7.34
Mannose	1.66
Isoprimeverose	3.22
Xylobiose	2.47
Cellobiose	6.80
Galacturonic acid	3.73
Glucuronic acid	0.19

Proteins are non-soluble in supercritical carbon dioxide; therefore, when lipids were extracted, its content increased from 15.2% to 17.9%. Lipid content decreased from 5% to 1.7%, as expected. Ash, lignin, and phenolic contents in TW powder are, respectively, 5.2%,19.2% and 0.57%, having a small variation after lipid extraction. Unlike carbohydrates, phenolic compounds were not qualitatively identified in this study with HPLC, although it is reported in the literature that caffeic acid, p-coumaric acid, chlorogenic acid, ferulic acid, gallic acid, quercetin, catechin, naringenin, rosmarinic acid, and rutin are some of the phenolic compounds found in tomato waste. [28], [29] Such identification should be performed in the future, to have a broader view of the possible valorization and applications of tomato waste. Such results are concordant with previous results found in literature. Table 3.4 summarizes the results of tomato waste characterization obtained in different studies.

Table 3.4: Tomato waste characterization in different studies.

Component	g $_{component}$ /100 g $_{powder}$						
	[25]	[53]	[57]	[58]	[59]	[60]	TW powder
% Peel % Seed	Mixture	1% 99%	Mixture	Mixture	77.8% 22.2%	100% 0%	Mixture
Protein	20.06	32.69	19.95	15.50	17.60	10.50	16.37
Ash	6.08	3.55	4.06	3.93	4.21	5.90	5.58
Lipids	13.04	15.43	10.67	6.30	2.19	4.04	5.37
Total Carbohydrates	n.a.	43.31	10.62	25.40	n.a.	n.a.	40.99
Dietary Fiber	28.01	29.42	55.10	38.00	52.44	n.a.	n.a.
Lignin	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	20.69
Phenolic Compounds	n.a.	0.11	n.a.	n.a.	0.12	0.02	0.62
Water	n.a.	5.03	10.14	n.a.	5.35	n.a.	7.10

“n.a." means that values were not available; values given in dry weight basis

As referred before, tomato waste composition varies depending on its origin and peel to seed ratio; therefore, the different studies as well as the present work, can have different compositions.

3.2. Subcritical Water Extraction

3.2.1. Process Efficiency and Yield of ScW Extract

In this work, ScW extraction of different components of tomato waste was studied throughout different temperatures. Temperature has a high influence in extraction efficiency with ScW, and in this study, one set of experimental parameters was studied. Two ScW extractions were made, under the same operating conditions. Results obtained in both extractions were very similar and concordant, attesting the reproducibility of the apparatus.

The color of the samples recovered was studied during the extraction process. The first extracts were clear and a little yellow, and as the temperature increased, they became more yellow/brownish and presented turbidness. Figure 3.1 shows this evolution of color and turbidity with the increase of temperature. Seven different extracts were collected at different temperatures, each having the purpose to extract different compounds with ScW. Extract 1 was collected between room temperature to 50 ºC, extract 2 from 50 ºC to 140 ºC and extract 3 was collected for 30 minutes at a constant temperature of 140ºC. Extract 4 and extract 5 were collected from 140 ºC to 190 ºC and for 30 minutes at 190 ºC, respectively. Extract 6 was collected from 190 ºC to 250 ºC and extract 7 was collected for 30 min at 250 ºC. The first extract is the clearest and the last is the darkest and with more turbidity.

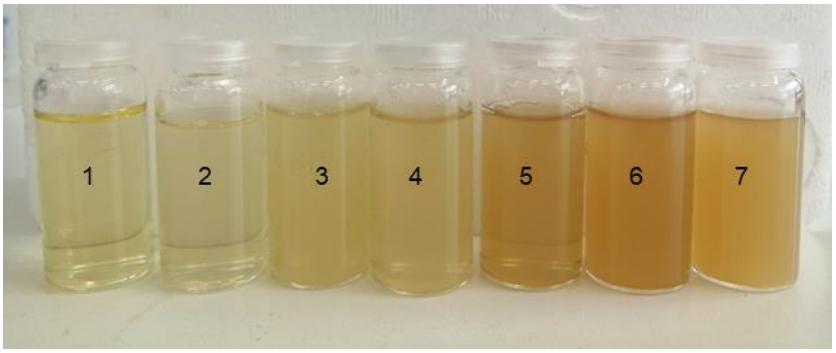

Figure 3.1: Extracts recovered from ScW extraction (Pressure= 100 bar; Flow rate= 12 mL/min).

Figure 3.2 represents the extraction temperature over time. To evaluate temperature influence in this extraction, a maximum temperature of 250 ºC was reached, under constant flow (12 mL/min) and pressure (100 bar), with a constant rise of temperature. Target temperatures for component extraction were 140 ºC, 190 ºC and its maximum at 250 ºC. It is clear in this figure the differences between the temperature stages at target temperatures for 30 minutes. Both assays were reproductible with success, under similar conditions.

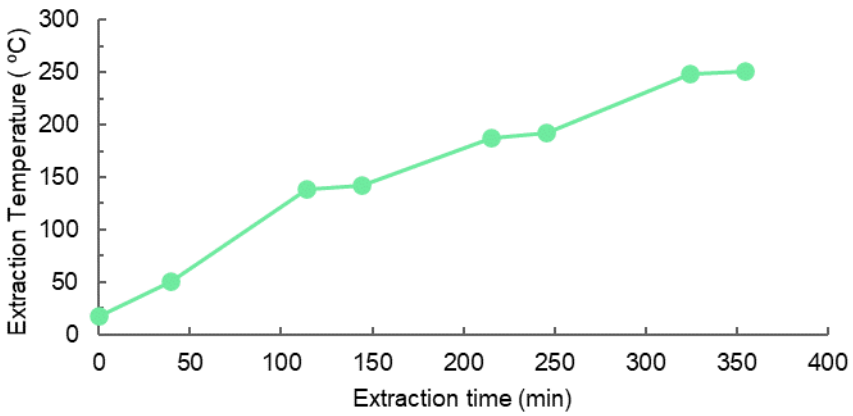

Figure 3.2: ScW extraction temperature over time.

Biomass conversion is given by Equation 3.1, where DTW $_{initial}$ is the amount of DTW placed in the reactor, in grams, before the extraction and DTW $_{final}$ is the amount left in the reactor, in grams, after the extraction.

$$\%\ Conversion = \frac{DTW_{initial} - DTW_{final}}{DTW_{initial}} \times 100 \qquad \text{Equation 3.1}$$

Extraction yield is given by Equation 3.2, where E is the total amount of ScW extract collected during the assay, after lyophilization, in grams, and DTW $_{initial}$ is the amount of DTW placed in the reactor, in grams.

$$\%\ Yield = \frac{E}{DTW_{initial}} \times 100 \qquad \text{Equation 3.2}$$

Extraction conditions, yield, and conversion are summarized in Table 3.5.

Biomass conversion and yield are expected to increase with temperature, because as the temperature increases, so does the ionic product of water, making it a stronger catalyst for the biomass hydrolysis. Also, the water viscosity decreases, increasing the mass transfer in the matrix of plant tissues. [34]

Extraction yield was 67%, and biomass conversion was 86%, both considerable high values. The duration of the assay can influence yield and biomass conversion, and the higher the maximum temperature, the longer the experiment takes, which can be an explanation for the high values obtained.

Table 3.5: ScW extraction conditions, yield, and conversion.

DTW $_{initial}$	Pressure	Water flow	% Yield	% Conversion	Maximum Temperature
18.54 g	100 bar	12 mL/min	67% ± 4.2%	86% ± 0.5%	250 ºC

Table 3.6 summarizes the extraction conditions, biomass extracted and yield throughout ScW extraction. The values are presented as an average of both experiments.

Table 3.6: ScW extraction conditions and results: cumulative mass (g) and cumulative yield (%) (Pressure = 100 bar; Flow Rate = 12 mL/min).

Extract	Extraction Time (min)	Temperature (ºC)	Cumulative Mass (g)	Cumulative Yield (%)
	0	17.3 ± 0.2		
1	39.5 ± 4.5	50.3 ± 0.3	6.4 ± 0.6	34.6 ± 3.2
2	114.0 ± 4.0	137.9 ± 1.2	8.5 ± 1.2	46.1 ± 3.3
3	144.0 ± 4.0	142.1 ± 0.7	9.3 ± 1.3	50.3 ± 3.6
4	215.5 ± 15.5	187.9 ± 1.3	10.3 ± 1.3	55.4 ± 3.8
5	245.5 ± 15.5	192.5 ± 1.3	10.8 ± 1.4	58.4 ± 4.1
6	324.5 ± 20.5	248.2 ± 1.1	11.9 ± 1.5	64.4 ± 4.1
7	354.5 ± 20.5	250.9 ± 0.2	12.4 ± 1.6	67.0 ± 4.2

-Values presented as an average of both experiments.

Figure 3.3 represents the extraction yield throughout the experiment. It is noticeable that extraction yield increases rapidly in the beginning, followed by stabilization near its maximum (67%). This figure suggests that up to 50 ºC (first marker) the extraction of non-structural sugars is the central process occurring, along with the extraction of a large portion of water-soluble proteins and phenolic compounds. In Table 3.1 it is clear that DTW is rich in non-structural carbohydrates, that are immediately available. This can explain the sudden increase of water-soluble compounds recovered in the beginning of the experiment. As temperature increases over 100 ºC (around 70 minutes) and enters the subcritical region, the extraction becomes slower, since polymers like hemicellulose are being hydrolyzed to release structural carbohydrates. Herewith, as water temperature increases, so does the ability to dissolve analytes and the penetrability of the matrix particles. [33] Also, ScW goes from polar to non-polar, promoting the dissolution of less polar compounds, such as polyphenols at higher temperatures. [36]

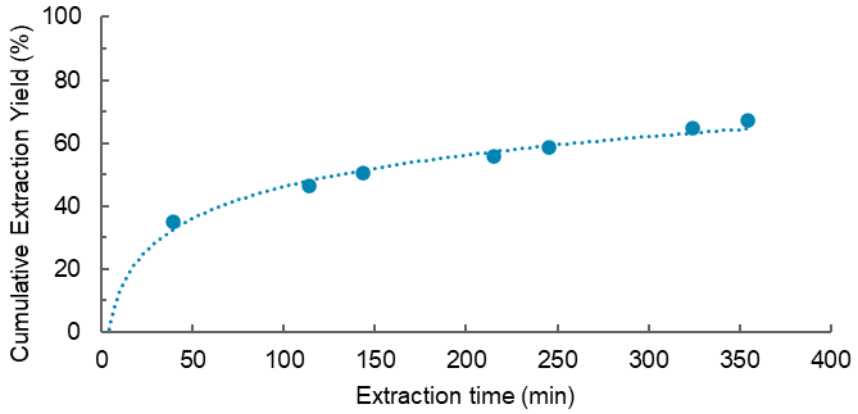

Figure 3.3: Kinetic reaction curve of the extraction yield throughout the experiment.

During the assay there is a 20.6% loss of biomass, that is not recovered from the reactor. This loss of biomass can be due to human errors, losses during the process, such as not taking all the ScW residue from the reactor, the cooling time, that takes about 1 hour, during which time some water-soluble compounds can be extracted but are not being considerate.

After characterization of each individual extract, these extracts were sorted according to the processing temperature. Extracts 1, 2 and 3 were grouped into one extract, called 140 ºC extract. Extracts 4 and 5 were grouped in the 190 ºC extract and extracts 6 and 7 in the 250 ºC extract. This is an important step to understand which compounds are being extracted in a certain range of temperature.

Table 3.7 summarizes the composition of the extracts, sorted according to the processing temperature. The values presented are an average of the values obtained in both experiments, except for protein and ashes. Protein content was determined with the extracts of the first experiment since there was not enough mass material for a replicate in the second experiment. Ash content, on the other hand, was only determined in the second experiment, for the same reason. This occurred because the volume of extraction samples was not sufficient in some cases for all the analysis required, considering the amount necessary for each method. However, as referred earlier, both experiments were reproductible and values obtained very similar.

Table 3.7: Composition of the extracts sorted according to the processing temperature.

Component	**g $_{component}$ /100 g $_{DTW\ powder}$**		
	140ºC Extract	**190ºC Extract**	**250ºC Extract**
Protein*	8.32 ± 1.73	3.56 ± 0.00	1.68 ± 0.00
Ash**	4.73 ± 0.41	0.68 ± 0.08	0.78 ± 0.05
Carbohydrates[1]	20.15 ± 1.64	1.32 ± 0.18	0.74 ± 0.12
Phenolic Compounds[2]	1.14 ± 0.20	0.68 ± 0.09	1.09 ± 0.12
Total	34.3 ± 4.0	6.2 ± 0.4	4.3 ± 0.3
Component	**g $_{component}$ /100 g $_{ScW\ extract}$**		
	140ºC Extract	**190ºC Extract**	**250ºC Extract**
Protein*	16.48 ± 0.78	43.19 ± 1.38	20.04 ± 0.64
Ash**	9.46 ± 0.82	8.54 ± 1.04	8.80 ± 0.59
Carbohydrates[1]	40.09 ± 3.30	16.20 ± 2.04	8.61 ± 1.31
Phenolic Compounds[2]	2.26 ± 0.40	8.37 ± 1.23	12.60 ± 1.06
Total	68.3 ± 5.3	76.3 ± 5.7	50.1 ± 3.6

*Values from the first extraction; **values from the second extraction;[1]Units in g GE /100 g $_{powder}$; [2] Units in g GAE /100 g $_{powder}$

In a grander overview, most compounds were extracted until 140 ºC, with special attention for carbohydrates, in which about 91% of total recovered carbohydrates, were recovered before

reaching this temperature. However, this does not apply to the phenolic compounds since their extraction is well distributed through the temperatures. Until 140 ºC, about 39% of the phenolic compounds were recovered, where in the 250 ºC extract there was about 37%.

Table 3.8 presents the composition of the reactor residue, which was characterized for protein, ash, carbohydrates, and lignin content. It is mostly composed of lignin and non-soluble carbohydrates and was also characterized for protein and ash content.

None of the extracts was fully characterized, unlike the ScW residue. The 250 ºC extract has only 50% of its components identified. A possible explanation is that during the ScW extraction a part of hemicellulose and cellulose can be extracted and solubilized in water, but does not hydrolyze completely, not accounting for sugar quantification. Another possible explanation is the presence of parts of free amino acids or peptides in the extracts, that are not quantified. Some other compounds are possibly not identified, that were formed during ScW extraction.

Table 3.8: Composition of the ScW residue obtained.

Component	**g $_{component}$ /100 g ScW residue**	**g $_{component}$ /100 g DTW powder**
Protein	2.48 ± 0.19	0.33 ± 0.03
Ash	7.66	1.03
Carbohydrates[1]	24.24 ± 1.77	3.27 ± 0.24
Lignin	62.73	8.46
Total	97.1 ± 2.0	13.1 ± 0.3

*Values from the first extraction; [1]Units in g GE /100 g $_{powder}$

In the following chapters, ScW extracts composition and the ScW residue left in the reactor will be studied with more detail.

3.2.2. Carbohydrate Content

As mentioned in Chapter 1.1.3, agro-industrial residues are mainly composed of lignocellulose, constituted by cellulose, hemicellulose, and lignin. Cellulose is a linear chain of glucose monomers linked by β-bonds, in a crystalline structure, making it difficult to hydrolyze into glucose. On the other hand, hemicellulose, with an amorphous structure, is more easily hydrolyzed to sugar monomers. [12], [13]

High temperature extraction increases the ability of water to solubilize analytes and hydrolyze various compounds. At temperatures above 160 ºC, ScW can solubilize hemicellulose and lignin. During a ScW extraction, part of the hemicellulose is hydrolyzed, forming acids, which will help to catalyze the hydrolysis of the rest of hemicellulose. [61]

As observed in chapter 3.1.1., tomato waste is a rich source of carbohydrates. Overall, 30.5% of DTW powder weight is composed of carbohydrates. Non-structural carbohydrates account for

15.1%, while structural carbohydrates are the remaining 15.4%, which are present as a part of the structure of cellulose and hemicellulose.

To assess the ability to extract/hydrolyze the carbohydrate fraction from DTW powder using ScW, carbohydrate quantification of ScW extracts recovered during the experiment was made, according to the methodology described in Chapter 2.3.1., using a D (+)-glucose monohydrate calibration curve. Carbohydrate content in each extract, in more detail than in Table 3.7 (which presents the extracts sorted by temperature), is presented in Table 3.9.

Most of carbohydrates were extracted in the first extract, until 50 ºC, which was already expected when analyzing the cumulative yield of the extraction in Figure 3.3. Progressively, the amount of carbohydrates extracted decreases with the increase of temperature. In extract 4, in the 140 to 190 ºC temperature range, there is a slight increase of carbohydrates extracted, as expected, since at temperatures above 160 ºC, ScW can solubilize hemicellulose, which will hydrolyze. Comparing the amount of carbohydrates extracted in this experiment with the total carbohydrate content quantified in the chapter 3.1.1, there is a 73% recovery of carbohydrates in these extracts (Figure 3.4).

Table 3.9: Carbohydrate content in each extract obtained at different temperatures.

Extract	Temperature range (ºC)	Carbohydrate content (g GE/ 100 g $_{SCW\ extract}$)			Carbohydrate content (g GE/ 100 g $_{DTW\ powder}$)		
1	T_{room} - 50	47.43	±	5.36	16.25	±	0.97
2	50 - 140	28.77	±	4.19	3.20	±	0.58
3	140	16.67	±	1.95	0.70	±	0.09
4	140 - 190	16.99	±	1.26	0.86	±	0.07
5	190	14.83	±	3.40	0.45	±	0.12
6	190 - 250	9.52	±	1.42	0.58	±	0.10
7	250	6.44	±	0.62	0.17	±	0.02
				Total	22.2	±	1.9

Figure 3.4 shows that the best condition for extraction/hydrolysis of carbohydrates is until 190 ºC. Reaching this temperature, the amount of carbohydrates extracted stabilizes, and the carbohydrate conversion from extract 5 (190 ºC) to extract 7 (250 ºC) is about 3%. A possible explanation is the degradation of monosaccharides at higher temperatures, and the non-hydrolyzation of cellulose, for which higher temperatures are necessary. If the purpose of this assay was to extract only carbohydrates, the experiment could have been interrupted at this point. However, other compounds, such as phenolic compounds require higher temperatures to be solubilized, due to their low polarity.

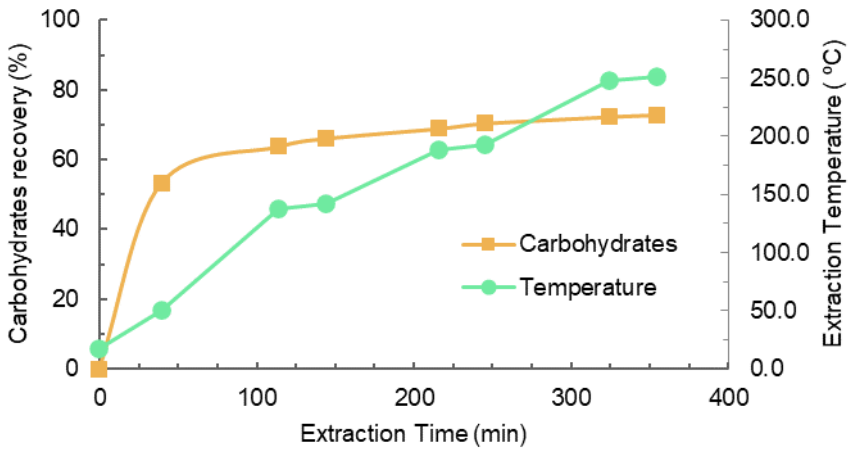

Figure 3.4: Cumulative carbohydrates recovered in the extracts, relative to the amount of DTW powder used in this experiment.

The carbohydrate quantification method is a colorimetric method and quantifies all carbohydrates from monosaccharides to polysaccharides. For a better understanding of the present carbohydrates in the ScW extracts, HPLC analysis was performed. Figure 3.5, concordantly with previous conclusions, shows that the best conditions for extraction of monosaccharides is until 140 ºC. In the first extract, until 50 ºC, glucose and fructose were detected in large quantities, while fucose, arabinose and galactose were detected in vestigial amounts, which represents about an 81% recovery of total monosaccharides found in extracts. In the second extract, from 50 ºC to 140 ºC, about 17% of total monosaccharides were recovered. Therefore, until 140 ºC about 98% of total monosaccharides were recovered. It was predictable that glucose and fructose would be present in these first extracts, since they exist in large amounts in the form of free sugars. The following extracts, with temperature increase, have low content of glucose and fructose, and hemicellulose starts hydrolyzing, with the appearance of fucose, rhamnose, arabinose, galactose, and xylose. Lastly, from 190 ºC, there are vestigial amounts of glucose, arabinose, xylose, galactose, and fructose. Once again, concordantly with previous conclusions, with temperature increase there could be degradation of monosaccharides.

One of the disadvantages of this extraction method is the co-extraction of compounds such as proteins, sugars and organic acids that might interfere in the phenolic compounds quantification, namely with the Folin-Ciocalteau method. [62] For this reason, solid phase extraction was performed to separate phenolic compounds from the rest of the components extracted, into two different fractions, with different applications.

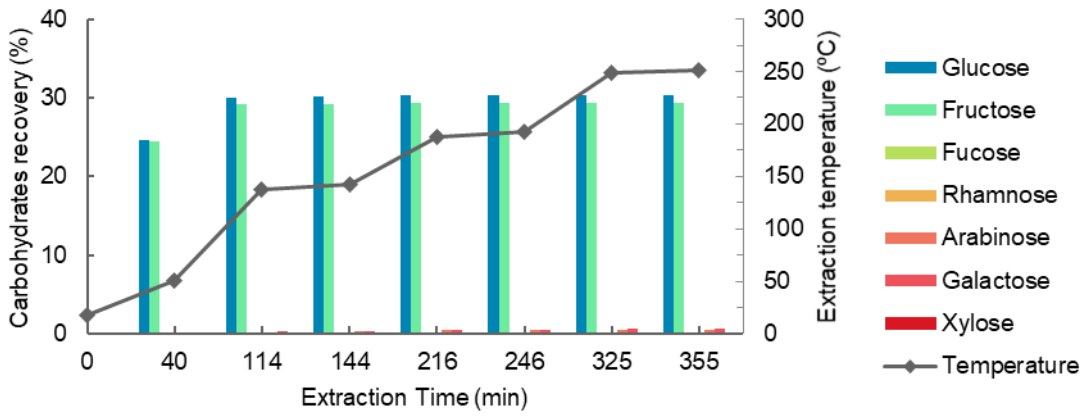

Figure 3.5: Cumulative monosaccharides recovered in the extracts, relative to the amount of DTW powder used in this experiment.

The sugar rich solution obtained after SPE was used for total carbohydrate quantification. Table 3.10 shows carbohydrate content of this solution. It is noticeable that the carbohydrate content is slightly lower than the carbohydrate content before SPE. One explanation for this is the possible presence of phenolic compounds in the carbohydrate quantification before SPE, that were then retained in the SPE column, lowering the total amount of carbohydrates in each extract. Another explanation is the degradation of carbohydrates with time since the carbohydrate quantification before SPE was performed earlier than the carbohydrate quantification after SPE. [63]

Table 3.10: Carbohydrate content in each extract after SPE.

Extract	Temperature range (ºC)	Carbohydrate content (g GE/ 100 g $_{ScW\ extract}$)			Carbohydrate content (g GE/ 100 g $_{DTW\ powder}$)		
1	T_{room} - 50	48.01	±	1.83	15.03	±	0.57
2	50 - 140	21.12	±	0.63	3.09	±	0.09
3	140	5.81	±	0.80	0.24	±	0.03
4	140 - 190	6.76	±	0.26	0.34	±	0.01
5	190	2.53	±	0.17	0.13	±	0.01
6	190 - 250	6.82	±	1.23	0.20	±	0.04
7	250	0.54	±	0.14	0.03	±	0.01
				Total	19.1	±	0.8

The reactor residue was also chemically characterized for carbohydrate content (Table 3.8). Non-soluble carbohydrate content was determined applying the total carbohydrate quantification method described in Chapter 2.3.1. using a D (+)-glucose monohydrate calibration curve. There is 11% of carbohydrates that were not extracted and were left in the reactor, comparing with the DTW powder carbohydrate content.

3.2.3. Phenolic Content

One of the main goals of this assay was to evaluate the extraction of phenolic compounds from tomato waste with subcritical water. In Chapter 1.1.4 it is noted that tomato contains a variety of phenolic compounds, specially from the flavonoids class. This class of compounds is known for its potent antioxidant activity, that can later be used in pharmaceutical, cosmetic and food industry, representing an essential role in potential health benefits. Previous studies have reported that caffeic acid, p-coumaric acid, chlorogenic acid, ferulic acid, gallic acid, quercetin, catechin, naringenin, rosmarinic acid, and rutin are some of the phenolic compounds that can be found in tomato waste, as referred earlier. The extraction of phenolic compounds is influenced by several factors, such as flow rate, extraction temperature, particle size, extraction time, etc. [64] During the extraction of polyphenols with ScW, there is thermal degradation and selective polyphenol extraction, which depend highly on extraction temperature and duration of the procedure. [65]

To assess the ability to extract/hydrolyze the phenolic compound fraction from DTW powder using ScW, the quantification of total phenolic compounds recovered during the experiment was performed, using the Folin-Ciocalteau method described as in Chapter 2.3.2, using a gallic acid calibration curve. Phenolic content in each extract is presented in Table 3.11. Most extracts have similar total phenolic content; yet each extract gets richer in phenolic compounds with the increase of the temperature, confirming that higher temperatures lead to extracts richer in phenolic compounds. The highest total phenolic content is in extract 6, in the 190 ºC to 250 ºC temperature range, with about 7.6 mg/g of DTW powder. This value is already much larger than the total phenolic content identified in Chapter 3.1.1. (Table 3.1). Overall, about 29 mg/g of DTW powder of phenolic compounds were extracted with ScW, which means that more phenolic compounds were extracted in the ScW extraction. (Figure 3.6).

Table 3.11: Total phenolic content in each extract.

Extract	Temperature range (ºC)	Phenolic content (g GAE/ 100 g $_{SCW\ extract}$)			Phenolic content (g GAE/ 100 g $_{DTW\ powder}$)		
1	T_{room} - 50	1.27	±	0.04	0.44	±	0.05
2	50 - 140	4.06	±	0.33	0.46	±	0.11
3	140	5.69	±	1.10	0.24	±	0.04
4	140 - 190	8.70	±	1.46	0.44	±	0.07
5	190	7.81	±	0.84	0.24	±	0.02
6	190 - 250	12.54	±	1.14	0.76	±	0.11
7	250	12.73	±	0.75	0.33	±	0.01
				Total	2.9	±	0.4

This difference can be explained by the lower efficiency of the hydro-alcoholic extraction, described in Chapter 2.2.6, since part of the phenolic compounds are trapped in the lignocellulosic structure, making its extraction more difficult. Another probable explanation is that during the ScW

extraction, the lignocellulosic structure is hydrolyzed, making non-structural phenolic compounds more accessible. Moreover, during the extraction there can be lignin degradation, one of the main components of lignocellulose, which is rich in polyphenols. This is suggested when carefully analyzing the lignin content obtained in the ScW residue.

Lignin content was determined using the methodology described in chapter 2.2.5. There are 8.46 g/ 100 g of DTW powder of lignin in the ScW residue, that, when comparing with the lignin quantified in the DTW powder, means that 44% of the lignin resisted to ScW treatment.

Figure 3.6 shows the evolution of total phenolic content over time and as a function of temperature, and that the extraction of phenolic compounds is directly linked with the temperature, since the extraction of these compounds closely follows the temperature increase.

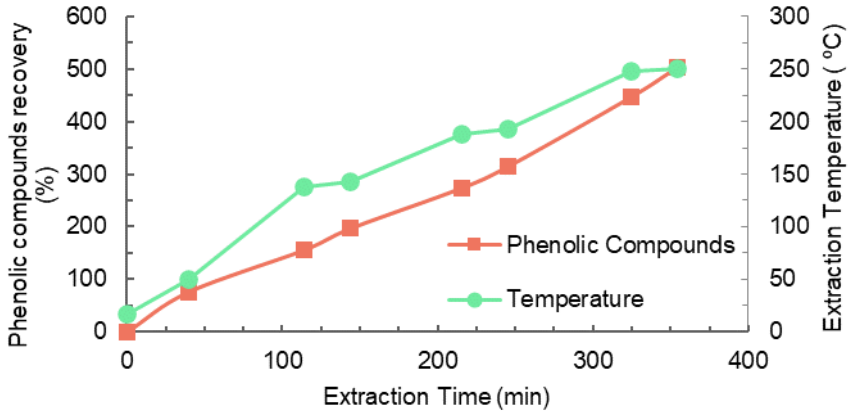

Figure 3.6: Cumulative phenolic compounds recovered in the extracts, relative to the amount of DTW powder used in this experiment.

Results obtained for phenolic compounds in this method are superior to the phenolic content described by other authors (Table 3.12). These other studies have reported the presence of phenolic acids such as caffeic, procatchoic, vanillic, catechin, gallic, chlorogenic, syringic, coumaric, salicylic and ellagic acids.

Table 3.12: Tomato waste phenolic content in different studies.

Component	**mg phenolic compounds /100 g powder**					
	[53]	[59]	[60]	[66]	**TW powder**	**ScW extracts**
% Peel **% Seed**	1% 99%	77.8% 22.2%	100% 0%	Mixture	Mixture	Mixture
Phenolic Compounds	109.57	122.95	16.16	148-202.2	616.27	2902.03

As it was referred in Chapter 3.2.2., the co-extraction of several DTW components interferes with the phenolic compounds quantification, when using the Folin-Ciocalteau method. Therefore, the phenolic compounds were extracted from the ScW extracts, using the SPE methodology

described in Chapter 2.5.1. Figure 3.7 shows the color evolution of the phenolic rich methanolic solutions obtained after SPE.

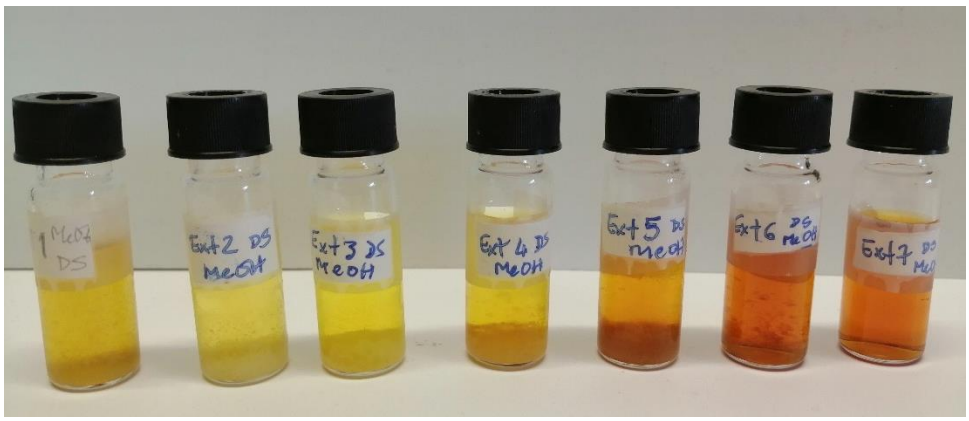

Figure 3.7: Phenolic rich methanolic solutions obtained after SPE.

Phenolic compounds of the phenolic rich methanolic solution were quantified using the Folin-Ciocalteau method, described in Chapter 2.5.3. Table 3.13 shows the total phenolic content in this methanolic solution. It is noticeable that the total phenolic content in the ScW extracts is considerably higher than the phenolic content in the methanolic solutions. One explanation for this might be the retention of bulkier phenolic compounds in the SPE column, that were not eluted with methanol. Additionally, the Folin-Ciocalteau method, a colorimetric method, is based on the color acquired when in contact with the Folin-Ciocalteau reagent, and it is not specific for phenols, since a reducing agent can be identified by this test. [67] However, even with the decrease of total phenolic content, the conversion of phenolic compounds is 294%. As suspected earlier, the lower efficiency of the hydro-alcoholic extraction, the lignin degradation or the lignocellulosic structure degradation can be the cause for this disparity of values.

Table 3.13: Total phenolic content in each extract after SPE.

Extract	Temperature range (ºC)	Phenolic content (g GAE/ 100 g $_{DTW\ powder}$)		
1	T_{room} - 50	0.15	±	7.2 x10^{-4}
2	50 - 140	0.29	±	9.5 x10^{-4}
3	140	0.11	±	2.5 x10^{-4}
4	140 - 190	0.34	±	7.8 x10^{-4}
5	190	0.14	±	4.1 x10^{-4}
6	190 - 250	0.47	±	4.9 x10^{-4}
7	250	0.21	±	5.1 x10^{-4}
	Total	1.7	±	4.1 x10^{-3}

Flavonoids are a group of phenolic compounds, that comprises a large range of phenolic compounds. These compounds are the most abundant phenolic compounds present in fruits and presents a more complex structure than phenolic acids.

Flavonoid content was determined with the colorimetric method described in Chapter 2.5.2, using a chatequin calibration curve. Table 3.14 shows the flavonoid content in each extract. About 12% of total phenolic compounds present in the extracts belong to the flavonoid group.

Table 3.14: Total flavonoid content in each extract after SPE.

Extract	Temperature range (ºC)	Flavonoid content (mg CE/ g $_{DTW\ powder}$)
1	T_{room} - 50	0.80 ± 0.01
2	50 - 140	0.78 ± 0.02
3	140	0.17 ± 0.01
4	140 - 190	0.40 ± 0.02
5	190	0.21 ± 0.01
6	190 - 250	0.76 ± 0.01
7	250	0.37 ± 0.01
	Total	3.5 ± 0.1

Like total phenolic content, the results obtained, for flavonoid content, in the extraction with ScW are higher than results obtained by other authors described in the literature, which have reported the presence of flavonoids such as myricetin, rutin, naringenin and quercetin (Table 3.15).

Table 3.15: Total flavonoid content in in other studies.

Component	mg $_{Flavonoids}$ /100 g			
	[53]	[59]	[66]	ScW extracts
% Peel	1%	77.8%	Mixture	Mixture
% Seed	99%	22.2%		
Flavonoid Content	68.82	41.53	22.0 - 41.54	349.11

Figure 3.8 shows the evolution of flavonoids extraction over time and as a function of temperature. The extract with higher flavonoid content is the first extract, until 50 ºC, with 0.8 mg per gram of DTW powder. The extraction of flavonoids is constant over time and does not stabilize throughout the entire experiment. This could be an indicator that if the temperature was kept constant at 250 ºC for a longer period, more flavonoids could be extracted.

As expected, phenolic compound extraction with ScW is favored, due to the lower polarity of the water, that increases the solubility of such compounds, and also the hydrolysis of the lignocellulosic structure that allows availability of a higher amount of phenolic compounds.

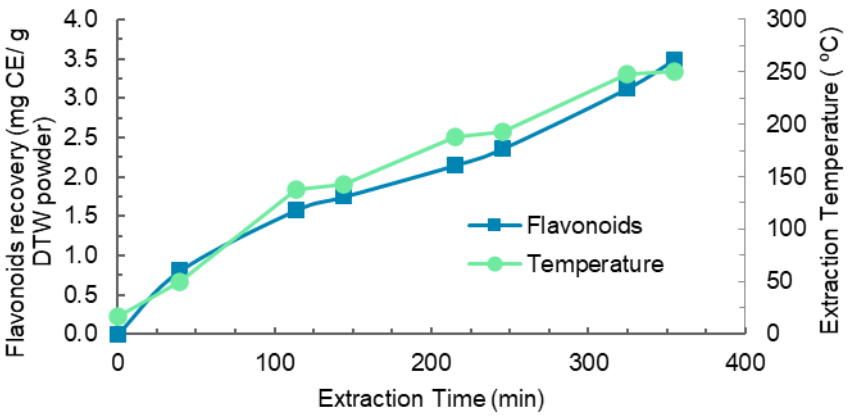

Figure 3.8: Cumulative flavonoids recovered in the extracts, over time.

3.2.4. Antioxidant Activity with DPPH

The antioxidant activity of ScW extracts (after SPE) was measured as described in Chapter 2.5.4., using a Trolox calibration curve. This assay is based on measuring the loss of the purple color from DPPH after reacting with ScW extracts. Phenolic compounds are a natural source of antioxidants; therefore, it is expected that the extracts with higher concentrations of phenolic compounds have a higher antioxidant activity. According to the Chapter 3.2.3, the extract 7 is the richest in phenolic compounds.

The inhibition percentage was determined for every ScW extract (Table 3.16), in which the extract 7, at the maximum temperature (250 ºC), has the highest antioxidant activity, which is an indicative that ScW extraction at higher temperatures produces extracts with higher antioxidant activity. The extract 4, in the range of temperatures of 140 ºC to 190 ºC, has a phenolic content of 3.4 mg/ g of DTW powder and an inhibition percentage of 21.7%, while the extract 5, at 190 ºC, has a phenolic content of 1.4 mg/ g of DTW powder and an inhibition percentage of 27.4%. This indicates that the antioxidant activity is not only related with phenolic content, but with the distinctive phenolic compounds in each individual extract.

Table 3.16: Inhibition percentage of each extract.

Extract	Temperature range (ºC)	% Inhibition
1	T_{room} - 50	39.26 ± 0.30
2	50 - 140	24.21 ± 0.27
3	140	30.31 ± 0.15
4	140 - 190	21.66 ± 0.24
5	190	27.44 ± 0.15
6	190 - 250	36.18 ± 0.09
7	250	54.07 ± 0.15

3.2.5. Gas Chromatography- Mass Spectrometry (GC-MS)

Gas Chromatography- Mass Spectrometry (GC-MS) is an analytical method that allows to identify different compounds in a test sample. In this case, GM-MS (Focus GC, Polaris Q - Thermo) was used to identify compounds in the phenolic rich methanolic solutions. Samples were acidified to a pH of 2, and derivatized whit 1 mL of pyridine and 0.1 mL of a derivatizing agent, bis(trimethylsilyl)acetamide. Finally, the extracts were injected in a GC-MS analyzer. [68] To identify the different compounds the mass spectrums and retention time were compared with the National Institute of Standards and Technology and Wiley databases.

For each extract, the main compounds identified were selected. Data processing was still in progress, and for this reason most of the major peaks were not yet identified. Table 3.17 shows the compounds identified in all the extracts and the correspondent peaks. For a general approach, three to four of the major peaks identified in each extract were selected, and amongst these, phenolic compounds, carboxylic acids, and sugar acids were identified.

Table 3.17: Main compounds identified with GC-MS and its correspondent peaks.

Compound	Extracts						
	1	2	3	4	5	6	7
(±) -Naringenin, O, O'-bis(trimethylsilyl)-	-	3	3	-	-	-	-
1-(2-(Trimethoxysilyloxy)-1-cyclopenten-1-yl)-ethanone	1	-	-	-	-	-	-
1,2,3-Propanetricarboxylic acid, 2-[(trimethylsilyl)oxy]-, tris(trimethylsilyl) ester	-	1	-	-	-	-	-
1,2-benzenedicarbonitrile, 3,6-bis(acetyloxy)-4-(1,1-dimethylethyl)-	3	-	-	-	-	-	-
1H-Indole-3-acetic acid, 1-(trimethylsilyl)-, ethyl ester (CAS)	-	2	2	-	-	-	-
4-Penten-2-one, 3-methyl-3-(1-methylethyl)-5-(trimethylsilyl)-	-	-	-	2	-	-	-
5-Hydroxymethylfurfural	-	-	-	-	-	1	-
Arabinofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-	-	-	4	-	-	-	-
Benzaldehyde, 4-[(trimethylsilyl)oxy]-	-	-	-	-	-	3	3
Benzaldehyde, 4-hydroxy-	-	-	-	-	3	-	-
Benzoic acid trimethylsilyl ester	2	-	-	1	2	2	2
Benzoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester	-	-	1	-	-	-	-
Catechol, TMS derivative	-	-	-	-	1	-	1
Vanillin	-	-	-	3	-	-	-

Figure 3.9 , Figure 3.10 and Figure 3.11 show a partial chromatogram of the first, second, and third extract, respectively, and the main peaks identified. In the first extract (room temperature to 50 ºC) it was expected to find water soluble compounds, and small amounts of phenolic compounds. After GC-MS, it was detected the presence of benzoic acid which can be a part of the hydroxybenzoic acids, a group of phenolic acids. Benzeneacetic acid (also known as phenylacetic acid) was also detected, being a carboxylic acid derived from acetic acid. Although SPE extracted the phenolic fraction from the extracts, sugar acids such as glyceric and 2,3,4,5-tetrahydroxypentanoic acids were also detected.

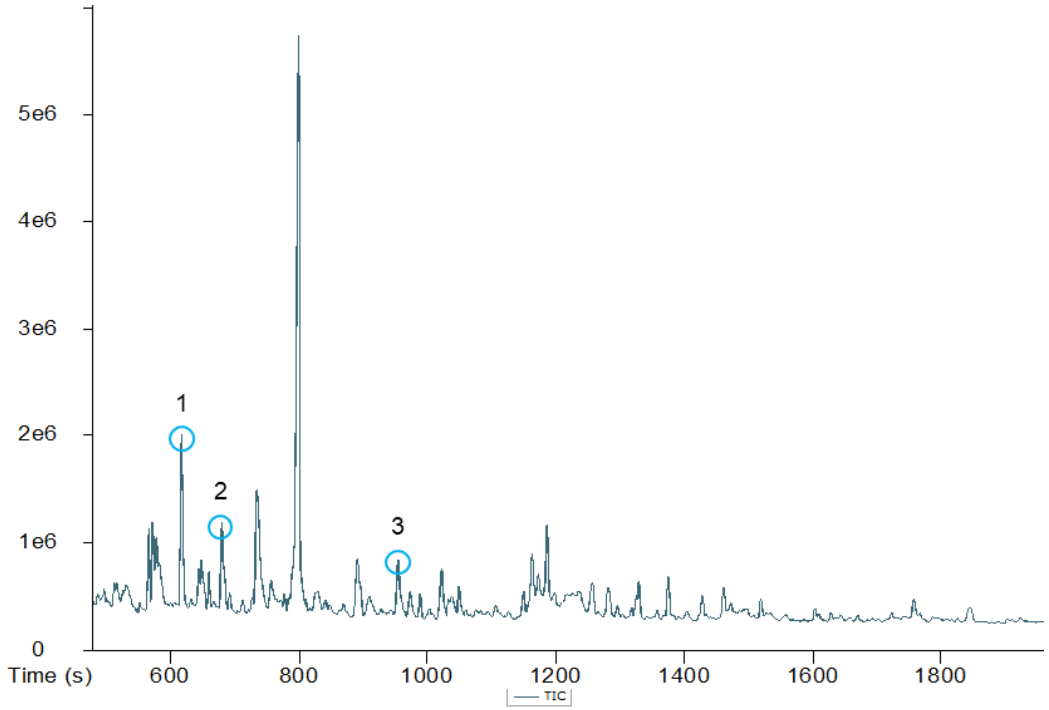

Figure 3.9: Extract 1 (room temperature to 50 ºC) chromatogram obtained from GC-MS. Peaks 1, 2 and 3 correspond to the identified compounds described in Table 3.17.

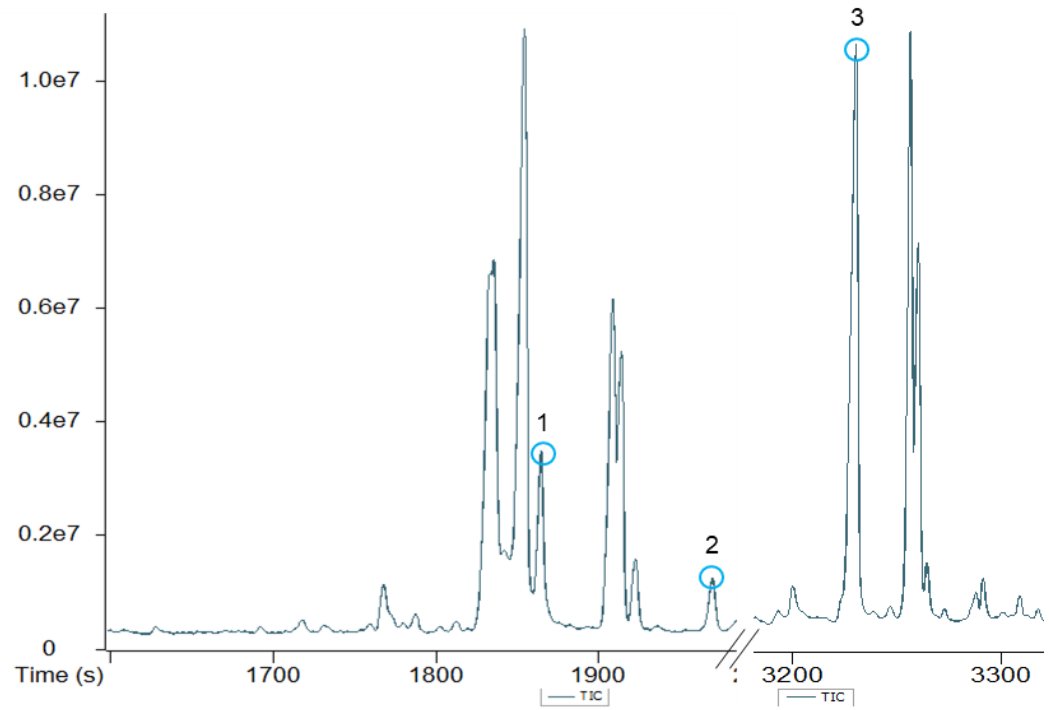

Figure 3.10: Extract 2 (50 to 140 ºC) chromatogram obtained from GC-MS. Peaks 1, 2 and 3 correspond to the identified compounds described in Table 3.17.

In the second extract (50 to 140 ºC) a diverse range of compounds were identified. Amino acids such as D,L-phenylalanine and pipecolic acid were identified, which confirms one of the possibilities that was discussed in Chapter 3.2.1., relatively to the incomplete characterization of

the ScW extracts. Additionally, cinnamic acid was detected in this extract, which is the central intermediate in the biosynthesis of lignin and lignocellulose precursors and flavonoids. A phenolic aldehyde, isovanillin was also detected. In both second and third extracts, naringenin, was identified in large quantities. Naringenin is a phenolic compound that is inserted in the flavonoids class. L-Proline and L-Aspartic acid are amino acids that were identified in these extracts. 1H-Indole-3-acetic acid is a hormone related to the vegetable's growth, and was identified in both extracts, as well as the azelaic acid, which is a carboxylic acid.

In addition to the compounds identified in both second and third extracts, the third extract (140 ºC) also included benzoic acid, which can be a part of the hydroxybenzoic acids, a group of phenolic acids. The increase of extraction temperature allowed, in these extracts, the extraction of several phenolic compounds and other important components.

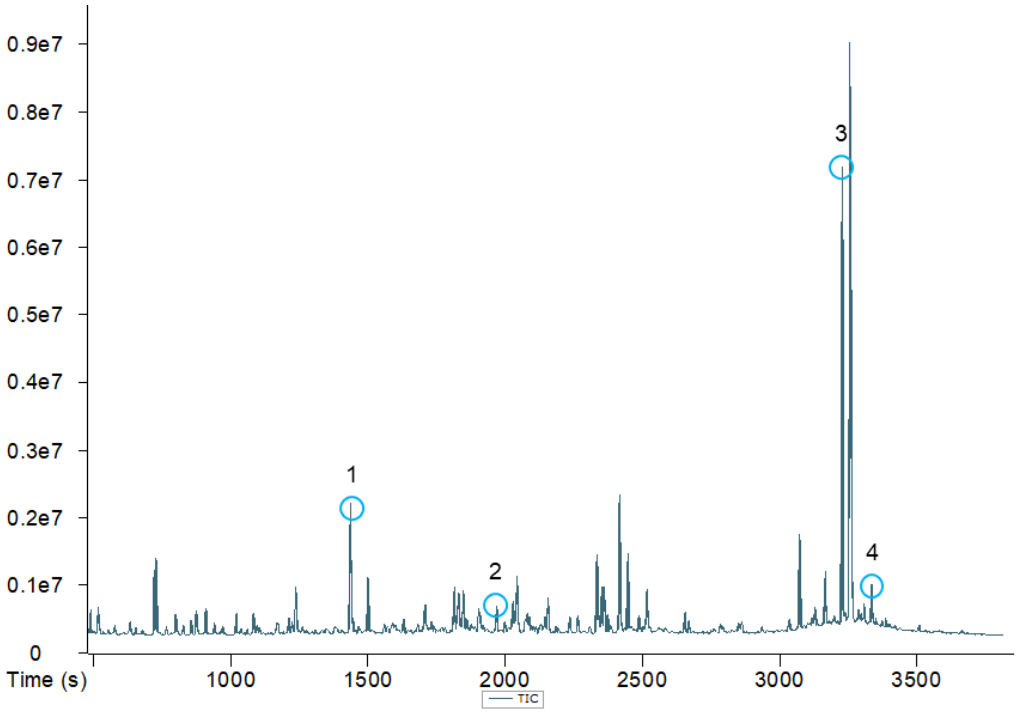

Figure 3.11: Extract 3 (140 ºC) chromatogram obtained from GC-MS. Peaks 1, 2 and 3 correspond to the identified compounds described in Table 3.17.

Figure 3.12 and Figure 3.13 presents the chromatograms for the fourth and fifth extracts, respectively, and the main peaks identified with GC-MS. These extracts comprise a range of temperatures from 140 ºC to 190 ºC, extracting several compounds with subcritical water. In the fourth extract, as well as the first and third extracts, benzoic acid was identified. In addition, a phenolic aldehyde, vanillin was also identified in large quantities. Diazomethane was also identified, and it is mainly used to convert carboxylic acids into methyl esters.

In the fifth extract, catechol, a thermal decomposition product of catechin, a phenolic compound, was identified. In addition, cyclic dipeptides such as cyclo (Pro-Leu) and cyclo(L-valyl-L-phenylalanyl) were identified, and can be accounted for the incomplete extracts' characterization,

as well as the amino acids present in the former extracts. Benzoic acid and acetone were also identified in this extract.

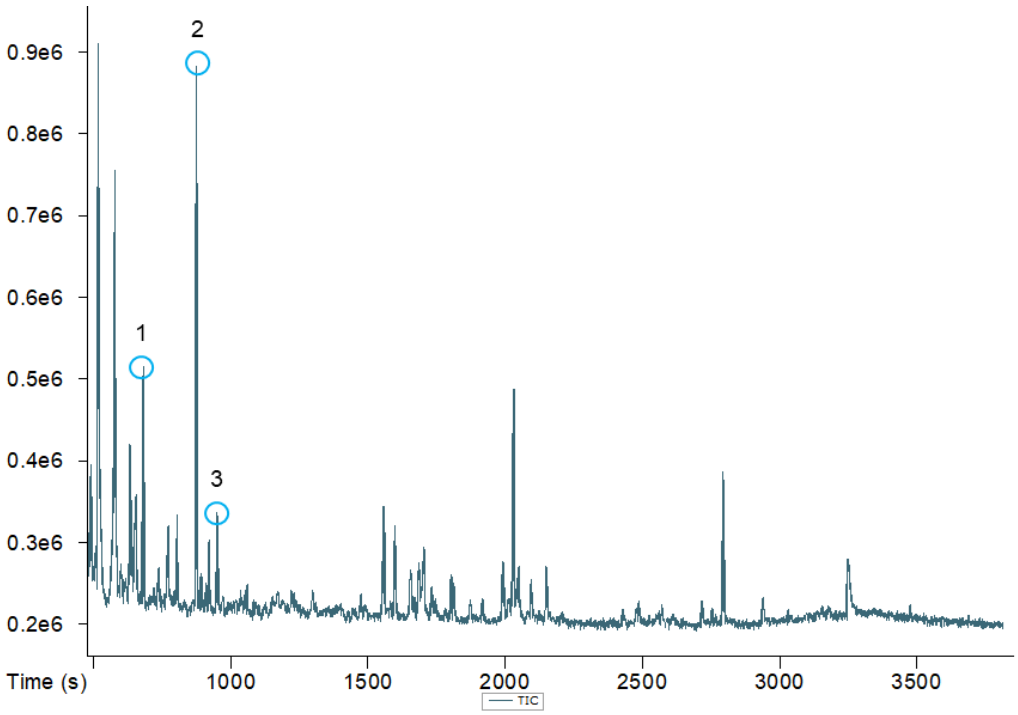

Figure 3.12: Extract 4 (140 to 190 ºC) chromatogram obtained from GC-MS. Peaks 1, 2 and 3 correspond to the identified compounds described in Table 3.17.

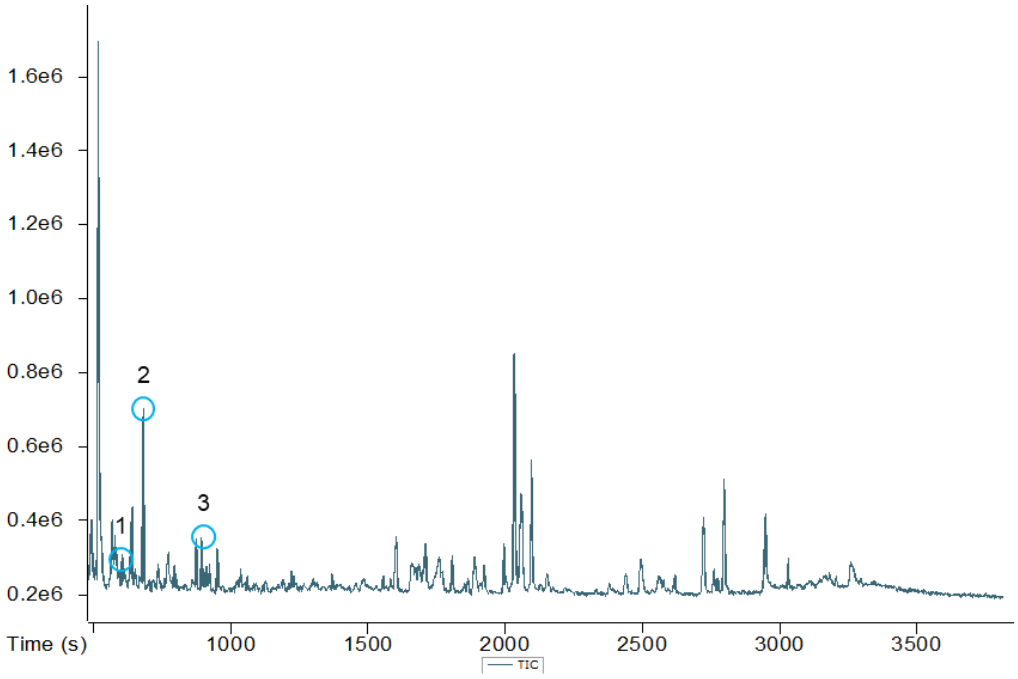

Figure 3.13: Extract 5 (190 ºC) chromatogram obtained from GC-MS. Peaks 1, 2 and 3 correspond to the identified compounds described in Table 3.17.

Figure 3.14 and Figure 3.15 shows the chromatograms for the sixth and seventh extracts, obtained with GC-MS, and the main peaks identified. These extracts comprise a range of

temperatures from 190 ºC to 250 ºC, and in this range there is the possible degradation of the lignocellulosic structure and hemicellulose. In these extracts, the less polar compounds are expected to be found. The sixth extract, as most of them contains benzoic acid, and similar to the extract five, contains cyclic dipeptides such as cyclo (Ala-Phe) and cyclo (Pro-Leu). It also contains hydroxymethylfurfural, a molecule that results from the transformation of monosaccharides such as fructose and glucose.

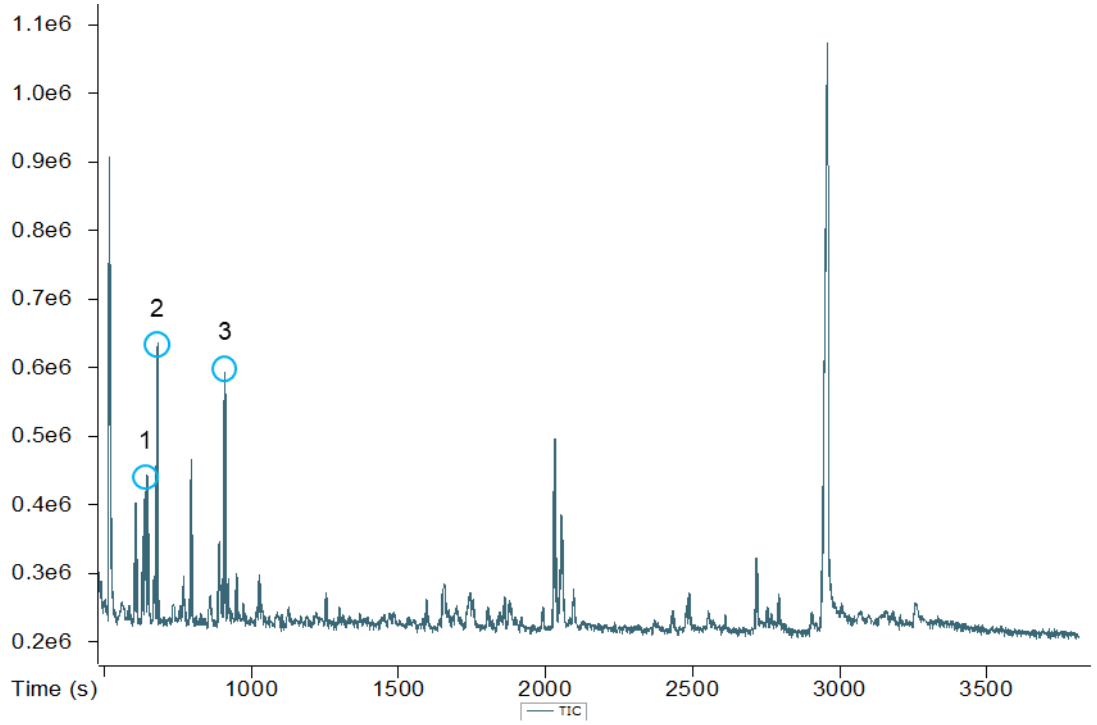

Figure 3.14: Extract 6 (190 to 250 ºC) chromatogram obtained from GC-MS. Peaks 1, 2 and 3 correspond to the identified compounds described in Table 3.17.

Finally, in the seventh extract catechol and benzoic acid were identified.

Benzaldehyde is also present in most extracts, whit the exception of the first and second extracts. This compound is the thermal decomposition product of a glycoside.

As representative compounds, present in most extracts, in Chapter 7.2 - Appendix in Figure 7.6 and Figure 7.7 are the mass spectrums of the benzoic acid and benzaldehyde, respectively. GC-MS allowed the identification of several compounds in each extract, especially phenolic compounds, such as naringenin, and phenolic aldehydes such as vanillin and isovanillin, allowing a greater view to which compounds are present at different extraction temperatures of the tomato waste.

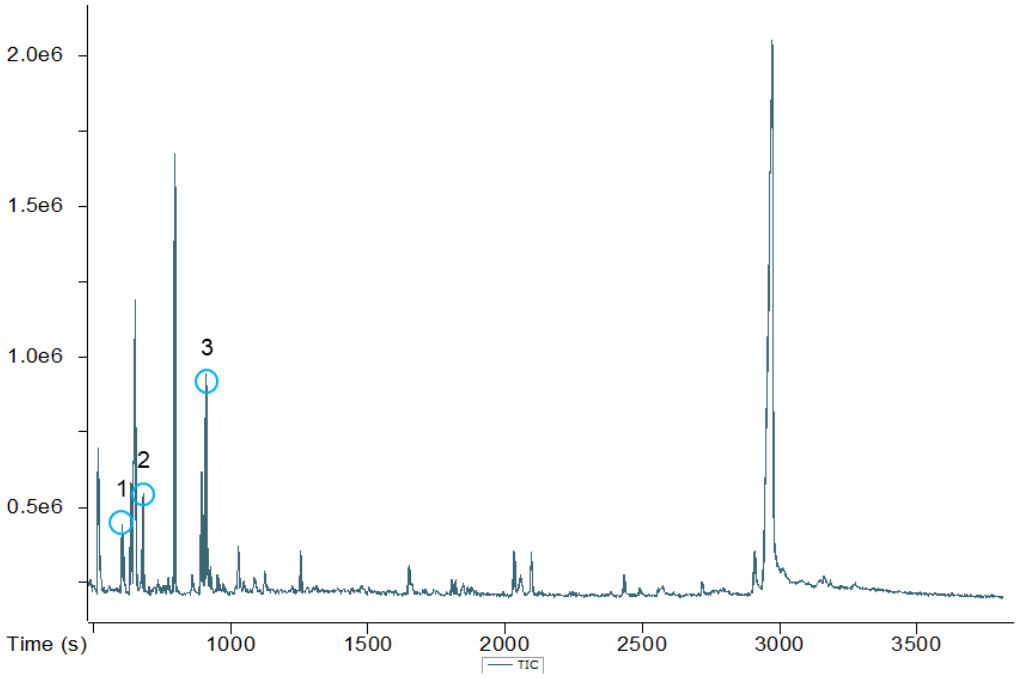

Figure 3.15: Extract 7 (250 ºC) chromatogram obtained from GC-MS. Peaks 1, 2 and 3 correspond to the identified compounds described in Table 3.17.

Chapter 4

Conclusions

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Conclusions

The main goal of this work was the valorization of tomato waste from tomato processing industries, using subcritical water for extraction/hydrolysis of valuable compounds, that can later be applied in several sectors.

Tomato waste was chemically characterized and noticed that it had a high carbohydrate content, of ca. 38 wt.%. In addition, glucose and fructose were identified as the main carbohydrate monomers in this residue. Besides carbohydrates, tomato waste was characterized for protein, ash, lipid, lignin content and phenolic compounds. Phenolic compounds are known for its potential antioxidant activity, and health benefits. Phenolic compounds content in tomato waste was ca. 0.6 wt.%.

To evaluate the influence of temperature in the extraction/hydrolysis with subcritical water, a set of parameters was studied throughout different target temperatures (140, 190 and 200 ºC). Two assays were carried out with a pressure of 100 bar and water flow of 12 mL/min, with a biomass conversion of 86% and an extraction yield of 67%.

Carbohydrate analysis of the extracts determined that the amount of carbohydrates extracted were 22.2 g/ 100 g of DTW powder, with a carbohydrate recovery of 73%. Most carbohydrates were recovered until 50 ºC, and progressively the amount of carbohydrates extracted stabilized above 190 ºC with temperature increase, making this the optimal temperature range conditions to extract carbohydrates. HPLC analysis shown that glucose and fructose are the main monosaccharides in the extracts, and up to 140 ºC about 98% of total monosaccharides are recovered. Other sugar monomers were also identified, such as fucose, rhamnose, arabinose, galactose, and xylose. Carbohydrate content analysis of the remaining solution after SPE fractionation of ScW extracts showed lower amounts of carbohydrates what can be due to retention of complex carbohydrates in the SPE column. Non-soluble carbohydrates were detected in the ScW residue, with 11% of recovery of carbohydrates, making up a total of 84% carbohydrate recovery in the process.

Phenolic content of the extracts was ca. 29 mg/g of DTW powder, which correspond to a higher amount then the quantified in the original material. Possible explanations for this may be a lower efficiency of the hydro-alcoholic extraction, the lignin degradation (that is composed of polyphenols) in the process, or the lignocellulosic structure degradation. Lignin degradation is supported by its low content in the ScW residue, where only 44% was recovered. The extraction of phenolic compounds is closely linked with the temperature of the process, and the extract that is most rich in phenolic compounds is the extract at the highest temperature. Phenolic compounds quantification after SPE was performed, with, as in carbohydrates, lower contents than before SPE. The retention of bulky phenolic compounds in the SPE column and the possible quantification of reducing agents when applying the Folin-Ciocalteau methodology are two possible causes for this decrease. Moreover, the total flavonoid, a large group of phenolic

compounds, content was determined, where about 20% of total phenolic content extracted belongs to this group. As expected, phenolic compound extraction with ScW is favored, due to the lower polarity of the water, that increases the solubility of those compounds and the hydrolysis of the lignocellulosic structure that allows availability of a higher amount of phenolic compounds available.

To evaluate the potential of tomato waste extracts, their antioxidant activity was evaluated. Two main conclusions can be taken: the extract with a higher antioxidant activity was obtained at the highest temperature studied (250 ºC) with an inhibition of 54% and the antioxidant activity of the extracts is intrinsically related not only with the nature of the phenolic compounds, but also with their concentrations in the different extracts.

The qualitative analysis of the extracts showed the presence of phenolic derivatives that are expected to be present in the tomato waste matrix such as naringenin or vanillin, but as extraction temperature increases it is possible to detect growing concentrations of sugar derivatives and products of their thermal decomposition such as hydroxymethylfurfural or the 4-hydroxy-benzaldehyde [69]. These differences suggest that the use of high temperatures may lead to the formation of unwanted components with associated negative properties [70].

The use of an appropriate fractionation technique to separate phenolic components from sugar derivatives is also recommended to avoid the presence of those carbohydrate components that do not contribute to the antioxidant properties of the extracts and may increase their susceptibility to microbial degradation.

Generally, the main goals of this work were achieved with success. Carbohydrate and phenolic compounds were extracted successfully with subcritical water and with high biomass recovery, and the presence of antioxidant activity was confirmed in ScW extracts.

Chapter 5

Future Work

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Future Work

To carry on the valorization of the residue studied in this assay, it is necessary to expand the knowledge acquired so far. Future work for this assay includes:

- Optimization of ScW extraction conditions for tomato waste extraction/hydrolysis, experimenting different target temperatures and different water flow rates. The yield and biomass conversion obtained are already high, but perhaps, with different extraction conditions the extraction can be more efficient.

- Study the composition of different tomato wastes, since the phenolic and carbohydrate composition changes according to the tomato origin and-study the detailed composition of tomato seeds, tomato pulp and tomato peels, to know what chemical composition is expected when tomato waste in composed, for example, by 30% peels, 50% pulp and 20% seeds. That way each tomato waste from different origins is more easily forwarded to a given application.

- More thorough identification of carbohydrates and phenolic compounds present in tomato waste.

- Deepen antioxidant activity analysis for ScW extracts and develop an application in the pharmaceutical, cosmetic, or food sector.

- Perform microorganism growth studies with ScW and evaluate its potential as an alternative carbon source or discover another application by producing added-value products from microbial activity. Perform antimicrobial activity tests with ScW extracts

- Optimization of extract upgrading methods to separate sugars from phenolic compounds in order to reduce sugar concentration in the final extracts.

After optimization of ScW extraction conditions for TW, and extract upgrading procedures, a scale up study should be taken into consideration.

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Chapter 6

References

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Chapter 7

Appendix

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Appendix

7.1. Calibration curves

7.1.1. Total Carbohydrate Quantification

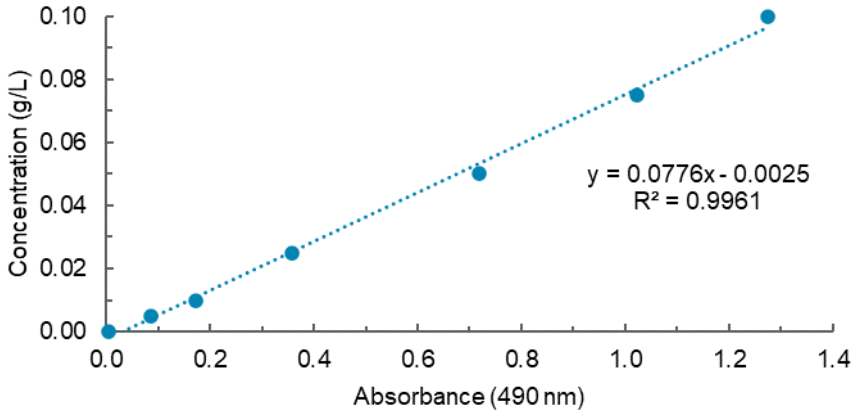

Figure 7.1: D (+)-glucose monohydrate calibration curve for total carbohydrate quantification

7.1.2. Total Phenolic Quantification – Folin-Ciocalteau Method

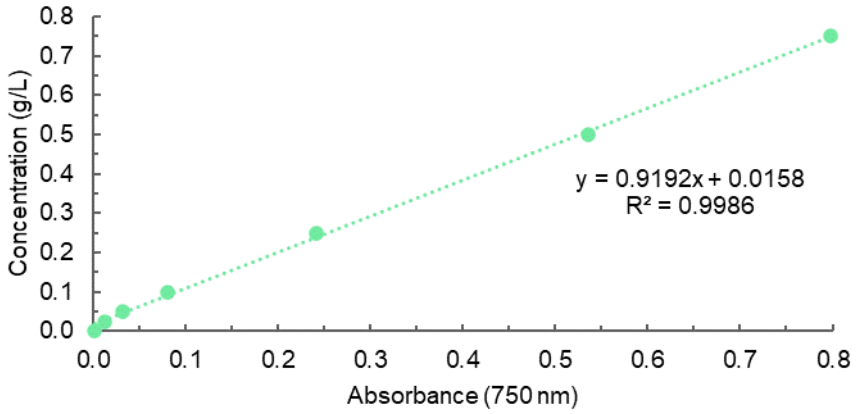

Figure 7.2: Gallic Acid calibration curve for total phenolic quantification

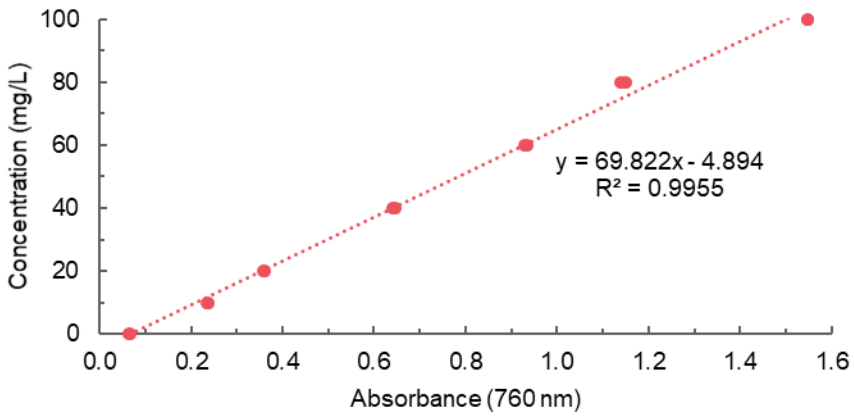

Figure 7.3: Gallic Acid calibration curve for total phenolic quantification after SPE

7.1.3. Total Flavonoid Quantification

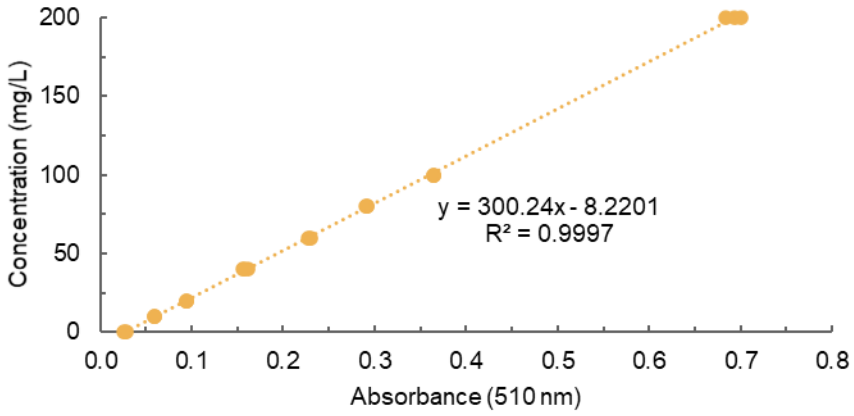

Figure 7.4: Catechin calibration curve for total flavonoid quantification after SPE

7.1.4. Antioxidant Activity with DPPH

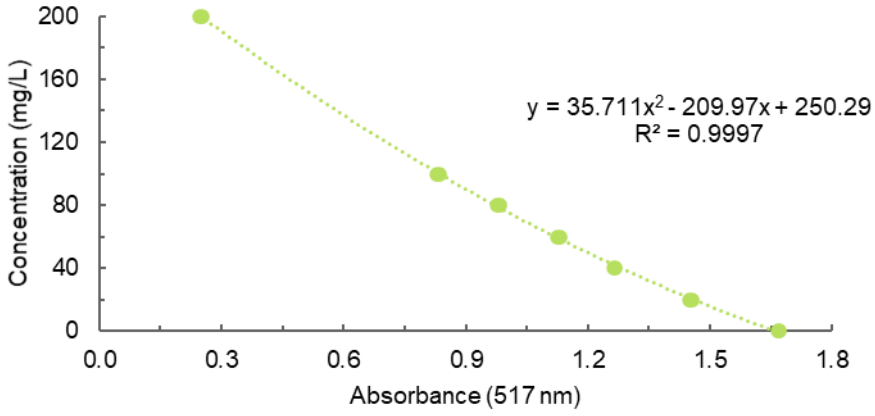

Figure 7.5: Trolox calibration curve for antioxidant activity with DPPH

7.2. Gas Chromatography- Mass Spectrometry (GC-MS)

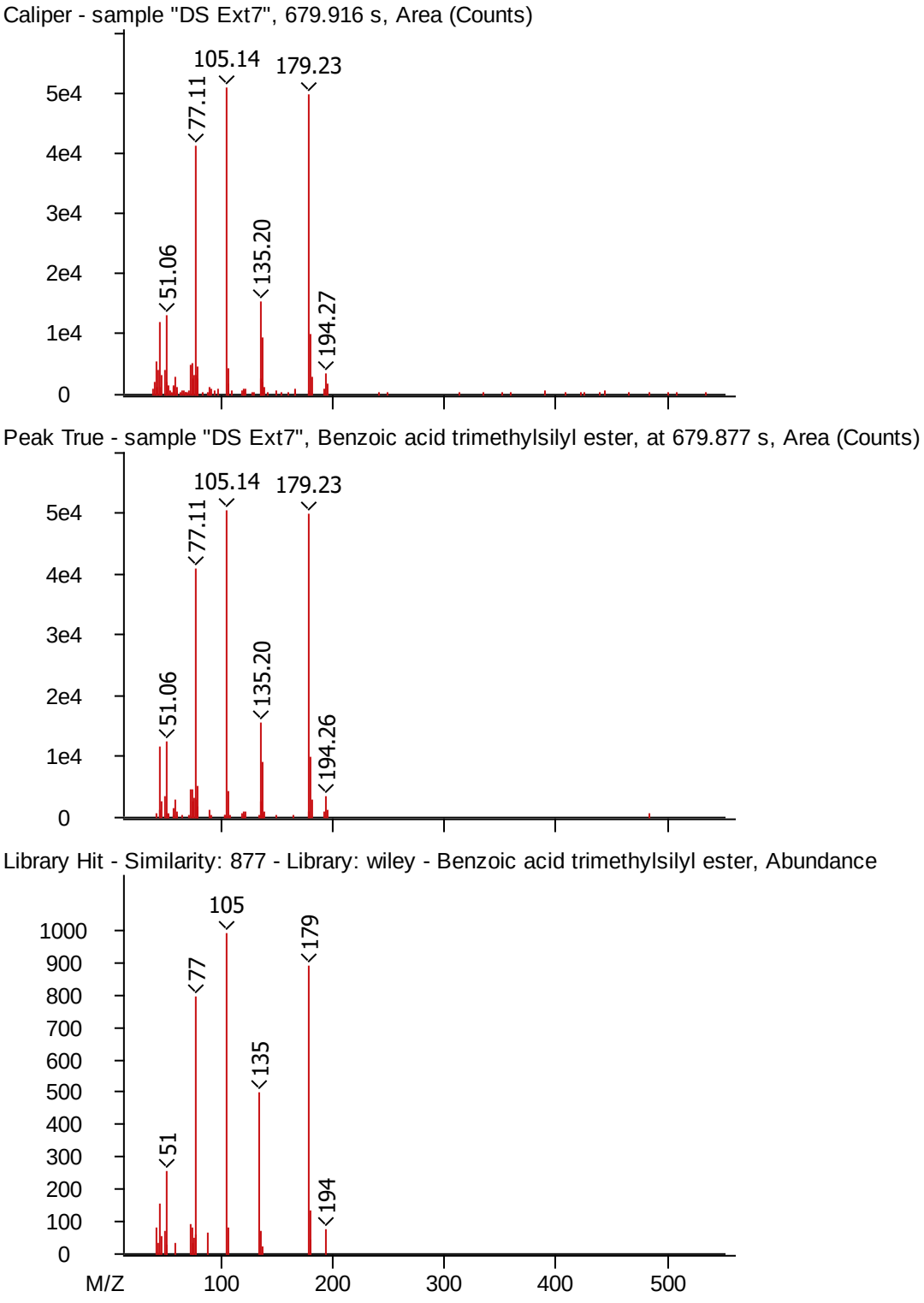

Figure 7.6: Mass spectrum of benzoic acid trimethylsilyl ester.

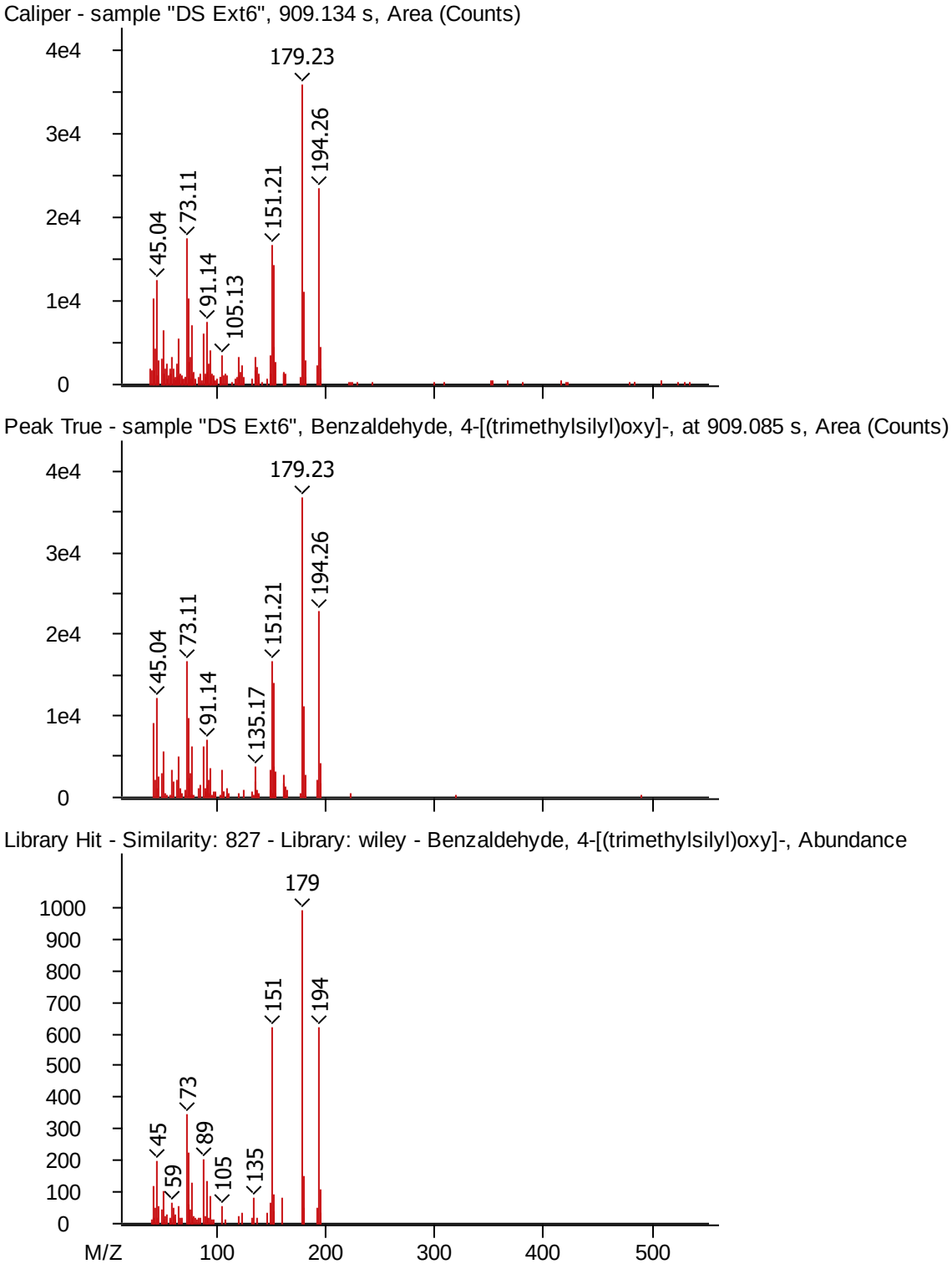

Figure 7.7: Mass spectrum of Benzaldehyde, 4-[(trimethylsilyl)oxy]-